Structural plasticity and post-translational modifications of C/EBPβ direct distinct myeloid cell fates

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Abstract

The CCAAT enhancer binding protein beta (C/EBPβ) transcription factor regulates differentiation, proliferation, and functionality of many cell types, including various cells of the immune system. A detailed molecular understanding of how C/EBPβ directs alternative cell fates remains largely elusive. Ectopic expression of C/EBPβ has been previously shown to reprogram committed B cell progenitors into inflammatory macrophages. We took advantage of this reprogramming system in order to examine how C/EBPβ regulates (trans)differentiation.

To determine which C/EBPβ protein modules are important for reprogramming, C/EBPβ wild type isoforms and mutants were ectopically expressed in primary mouse B cell progenitors. The data showed that the translationally regulated long isoforms LAP* and LAP, but not the N-terminally truncated isoform LIP can reprogram lymphoid cells into myeloid cells. Furthermore, we found that conserved regions 2,3 and 4 in the C/EBPβ protein transactivation domain are necessary and sufficient for B-to-myeloid cell conversion. Interestingly, the reprogrammed myeloid cells were found to represent a heterogeneous mixture of different myeloid cell types that express hallmarks of resident and inflammatory monocytes/macrophages, dendritic cells, and/or granulocytes. Detailed analyses of the reprogrammed $CD11b^+$ cells, based on myeloid differentiation marker expression, cell morphology, and gene expression profiling, revealed that discrete conserved regions in C/EBPβ activated distinct pro- and anti-inflammatory genes and triggered divergent differentiation programs. Moreover, not only structural C/EBPβ mutants, but also post-translational modification (PTM) site mutations led to different reprogramming outcomes. These data suggest that C/EBPβ orchestrates myeloid diversification by integrating PTMs with structural plasticity as signal dependent adaptable modular properties to determine cell fate.

Zusammenfassung

Der CCAAT enhancer binding protein beta (C/EBPβ) Transkriptionsfaktor reguliert die Differenzierung, Proliferation und Funktion vieler Zelltypen, einschließlich verschiedener Zellen des Immunsystems. Eine detaillierte molekulare Analyse des Mechanismus, wie C/EBPβ alternative Zellschicksale steuert, wurde jedoch bisher noch nicht unternommen. Es wurde gezeigt, dass die ektopische Expression von C/EBPβ in determinierten B- Vorläuferzellen diese zu inflammatorischen Makrophagen reprogrammieren kann. Wir haben dieses Reprogrammierungsystem verwendet, um die Strukturelemente in C/EBPβ, die für die Regulation der (Trans)Differenzierung durch C/EBPβ wichtig sind, zu untersuchen.

Um die maßgeblichen C/EBPβ Proteinmodule für die Reprogrammierung zu bestimmen, wurden entweder C/EBPβ Wildtyp Isoformen oder Mutanten in primären murinen B-Vorläuferzellen ektopisch exprimiert. Die Analysen ergaben, dass die translational regulierten langen Isoformen LAP* and LAP, jedoch nicht die kurze Isoform LIP lymphoide Zellen zu myeloischen Zellen reprogrammieren können. Des weiteren haben wir gezeigt, dass die konservierten Regionen 2, 3 und 4 der C/EBPβ Transaktivierungsdomäne essentiell und ausreichend für die Konvertierung von B Zellen zu myeloischen Zellen sind. Die reprogrammierten myeloischen Zellen setzten sich aus einer heterogenen Population verschiedener myeloischer Zelltypen zusammen, die typische Erkennungszeichen von residenten und inflammatorischen Monozyten/Makrophagen, dendritischen Zellen, und/oder Granulozyten aufwiesen. Detaillierte Analysen von $CD11b^+$ reprogrammierten Zellen, welche die Expression myeloischer Entwicklungsmarker und entsprechende Zellmorphologie aufwiesen, sowie für myeloische Genexpressionsmuster überprüft wurden, zeigten, dass diskrete konservierte Regionen von C/EBPβ verschiedene pro- und anti-inflammatorische Gene und divergente Entwicklungsprogramme aktivierten. Des Weiteren führten nicht nur strukturelle C/EBPβ Mutanten sondern auch Puktmutationen an Stellen, die posttranslationalen Modifikationen (PTM) unterliegen, zu verschiedenen Reprogrammierungsergebnissen. Diese Daten zeigen, dass die C/EBPβ abhängige myeloische Diversifikation durch die Integration von strukturellen C/EBPβ Proteinmodulen und deren signalabhängigen PTMs erreicht wird.

List of abbreviations[1]

°C	degree Celsius
A	alanine
AA	amino acid
ALP	all-lymphoid progenitor
APS	ammonium persulphate
BCR	B cell receptor
BLP	B cell-biased lymphoid progenitor
BM	bone marrow
bp	base pairs
BSA	bovine serum albumin
bZip	basic leucine zipper
CCL	chemokine (C-C motif) ligand
CD	cluster of differentiation
cDC	conventional dendritic cell
CDP	common dendritic cell progenitors
C/EBP	CCAAT enhancer binding protein
CFU	colony-forming unit
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
CR	conserved region
CXCL	chemokine (C-X-C motif) ligand
DAPI	4',6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
DC	dendritic cell
dpc	days post coitum
dwater	distilled water
EDTA	ethylenediaminetetraacetate
EPO	erythropoietin
ETP	early T cell progenitor

[1] Gene symbols are not included in the list

FACS	fluorescence activated cell sorting
Fig.	figure
Flt-3	fms-like thyrosine kinase 3 receptor
Flt-3L	fms-like thyrosine kinase 3 ligand
G-CSF	granulocyte colony stimulating factor
GFP	green fluorescent protein
GM-CSFR	granulocyte-macrophage colony stimulating factor receptor
GMP	granulocyte-macrophage progenitor
Gr	granulocyte
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hiFCS	heat inactivated fetal calf serum
HSC	hematopoietic stem cells
IFN	interferon
Ig	immunoglobulin
IgH	immunoglobulin heavy chain
IL	interleukin
iPS	induced pluripotent stem (cell)
IRES	internal ribosome entry site
IRF	interferon-regulatory factor
L	leucine
LAP	liver activating protein
LCR	low complexity regions
Lin	lineage
LIP	liver inhibitory protein
LMPP	lymphoid primed multipotent progenitor
LPS	lipopolysaccharide
LZ	leucine zipper
MAPK	mitogen-activated protein kinase
M-CSFR	macrophage colony stimulating factor receptor
MDP	macrophage and DC progenitor
MEF	mouse embryonic fibroblasts
MEP	megakaryocyte-erythrocyte progenitor
miRNA	microRNA
MPh	macrophages

MPP	multipotent progenitor
MSCV	murine stem cell virus
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
NK	natural killer
pDC	plasmacytoid dendritic cell
pre-BCR	pre-B cell receptor
Q	glutamine
R	arginine
RNA	ribonucleic acid
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PTM	post-translational modification
RD	regulatory domain
RT	room temperature
S	serine
SCF	stem cell factor
SEM	standard error of the mean
SUMO	small ubiquitin-like modifier
T	threonine
TAE	Tris/ acetate/ EDTA buffer
TAD	transactivation domain
TBST	Tris-Buffered Saline / Tween
Tris	2-amino-2-hydroxymethyl-propane-1,3-diol
T_H	T helper cell
Tip-DC	TNF-α and iNOS-producing dendritic cell
uORF	upstream open reading frame
URE	upstream regulatory element
WT	wild type
4Y	4 Yamanaka transcription factors

1. Introduction

The hematopoietic lineage specification depends on transcription factors that set lineage specific networks in different hematopoietic cells. Multiple transcription factors can act in sequential and synergetic manner or can antagonize each other to determine cell fate decisions (Fiedler and Brunner, 2012; Laiosa et al., 2006a; Laslo et al., 2008). Furthermore, the same transcription factor can be expressed in different cell types and may be involved in regulation of alternative cell fates (Dakic et al., 2007). Despite recent advances and extensive efforts to understand hematopoietic cell fate choices, combinatorial interactions between transcription regulators and integration of extracellular signals remain challenging tasks in developmental biology and molecular genetics. When ectopically expressed, some key transcription factors can perturb the established gene expression profiles setting new ones, which might lead to reprogramming of one hematopoietic cell type into another (Graf, 2011; Graf and Enver, 2009). Importantly, this transcription factor induced trans-differentiation of one committed cell type into another one has been achieved through enforced expression of lineage-determining transcription factors which have a central function in the physiological differentiation of the resultant cell type, suggesting that cell lineage reprogramming could be used to explore cell fate decision mechanisms and their regulation.

1.1. Hematopoiesis and hematopoietic lineage diversification

Hematopoiesis is the process of formation and differentiation of blood cells. Hematopoiesis is organized in a hierarchical manner, starting with the multipotent hematopoietic stem cells (HSCs) which reside in the bone marrow (BM) of the adult and give rise to lineage committed progenitors capable of terminal differentiation into mature cells. Within the HSC compartment there is a rare subpopulation of long-term HSCs which have the distinctive capability of life-long self-renewal and multilineage differentiation. They give rise to short-term HSCs, which keep the multilineage differentiation ability, however have a reduced self-renewal potential (Rosenbauer and Tenen, 2007). The classical model of hematopoiesis has suggested a mechanism of separate differentiation into two distinct lineage progenitors, namely common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs),

which are derived from a shared multipotent progenitor (MPP) completely lacking the self-renewal potential of the HSCs. CLPs can generate lymphoid lineage cells like natural killer (NK) cells, T cells, and B cells, while CMPs can further branch into two more restricted progenitors: megakaryocyte-erythrocyte progenitors (MEPs) and granulocyte (including neutrophils, eosinophils and basophils) -macrophage progenitors (GMPs) (Akashi et al., 2000; Kondo et al., 1997; Laiosa et al., 2006a). However, recent work has challenged this classical model due to the discovery of alternative differentiation pathways and additional intermediate progenitor steps (Graf, 2008; Murre, 2009) (Fig. 1.1). For example, Adolfsson et al. have shown that HSC can give rise of MEPs and lymphoid primed multipotent progenitors (LMPPs) which have lost erythroid and megakaryocytic developmental potential but retain myeloid and lymphoid potential, and this finding questions the concept of CMPs and the origin of MEPs and suggests that GMPs can be generated through alternative pathways (Adolfsson et al., 2005). Upon loss of myeloid potential LMPPs generate CLPs which are not a homogenous population and based on the expression of Ly-6D surface marker could be subdivided to Ly-6D$^-$ all-lymphoid progenitors (ALPs), which possess the full lymphoid potential and can differentiate into B, T, NK, and dendritic cells (DCs), and more restricted Ly-6D$^+$ B cell-biased lymphoid progenitors (BLPs) which upregulate the B cell specific transcription factors *Ebf1* and *Pax5* and preferentially develop along the B cell differentiation pathway via the pre-pro and pro B cell stages (Inlay et al., 2009). The pro B cells undergo B-lineage commitment, rearrange their immunoglobulin heavy chain (IgH) locus and successful rearrangements result in expression of the pre-B cell receptor (pre-BCR) on the cell surface and transition from the pro B to the pre B cell stage (Medvedovic et al., 2011). Productive immunoglobulin light chain gene rearrangements in pre B cells result in the development of immature surface IgM$^+$ B cells which leave the BM and migrate to the peripheral lymphoid organs (Medvedovic et al., 2011). Moreover, studies have shown that pre-pro B cells possess not only B cell differentiation capacity but also residual T cell potential (Rumfelt et al., 2006). Moreover, the early T cell progenitors (ETPs) have combined T cell, B cell and myeloid potential, indicating the existence of alternative lineage commitment pathways (Bell and Bhandoola, 2008; Luc et al., 2012).

Earlier *in vivo* transplantation experiments have shown that CLPs, ETPs, pro T cells, as well as CMPs and GMPs can generate DCs, whereas pro B cells and MEPs do

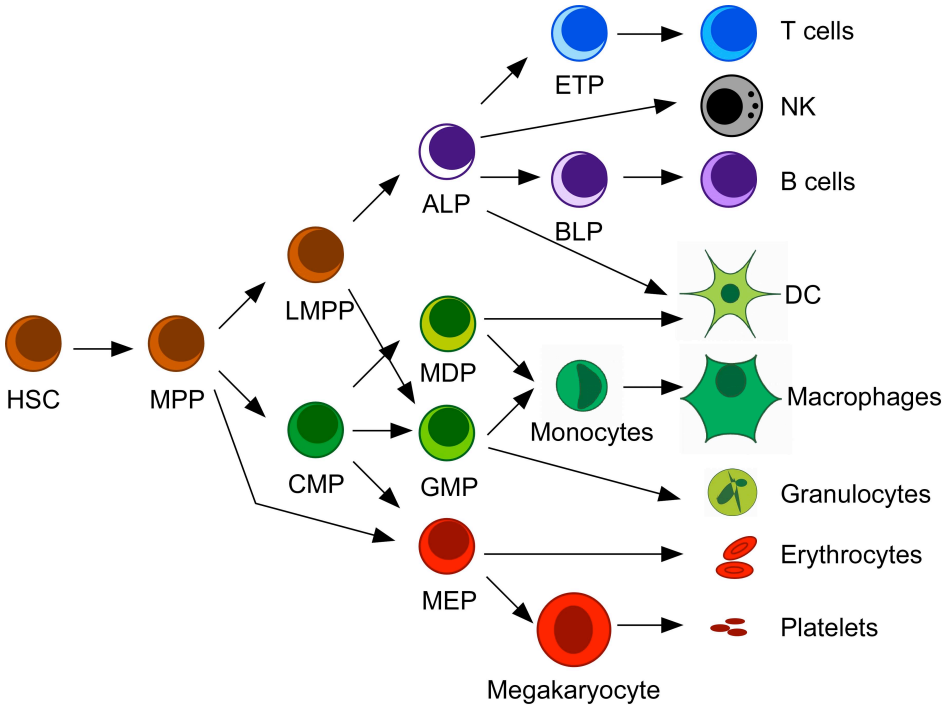


Fig. 1.1 Current concepts of hematopoietic lineage diversification.

The scheme represents a revised roadmap of the hematopoiesis, combining the classical model with recent advances in the hematopoietic cell development field, which have suggested the existence of alternative differentiation pathways and cell fate branching. For the sake of simplicity, only some but not all alternative paths are shown. See text for more details. HSC - hematopoietic stem cells; MMP - multipotent progenitors; LMPP - lymphoid primed multipotent progenitors; ALP - all-lymphoid progenitors; BLP - B cell-biased lymphoid progenitors; ETP - early T lineage progenitors; NK - natural killer cells; DC - dendritic cells; CMP - common myeloid progenitors; MDP - macrophages and DC progenitors; GMP - granulocyte-macrophage progenitors; MEP - megakaryocyte-erythrocyte progenitors.

Figure is based on published data (Adolfsson et al., 2005; Fiedler and Brunner, 2012; Fogg et al., 2006; Geissmann et al., 2010; Inlay et al., 2009; Ramirez et al., 2010; Rosenbauer and Tenen, 2007).

not have DC differentiation capacity; furthermore, CLPs and CMPs have approximately equal DC developmental potential on a per cell basis (Manz et al., 2001). Later, the existence of a common macrophage and DC progenitor (MDP) downstream of CMPs characterized as Lin^{-} c-kit^{+} $CX_3CR_1^{+}$ M-$CSFR^{+}$ population was identified and this MDP can *in vivo* differentiate into monocytes, different tissue macrophage populations and resident splenic DC but not into granulocytes (Fogg et al., 2006). DC development further progresses to common DC progenitors (CDP), which ultimately give rise to conventional DCs (cDCs) and plasmacytoid DCs (pDCs) but not to monocytes (Liu et al., 2009). On the other hand, blood monocytes originate

in the BM from HSCs via several progenitor stages including CMP, GMP and MDP (Yona and Jung, 2010). Although it is currently accepted that monocytes do not reconstitute cDCs and pDCs in the steady state, under inflammatory conditions they can enter the tissues and give rise not only to macrophages but also to inflammatory DCs, like for example the TNF-α and iNOS (Nos2)-producing (Tip) DCs identified in spleens of mice infected with Listeria monocytogenes (Geissmann et al., 2010; Serbina et al., 2008; Serbina et al., 2003). However, the existence of HSC independent myeloid lineage, including liver Kupffer cells, epidermal Langerhans cells and brain microglia, has been shown. These cells may originate from embryonic yolk sac progenitors and are maintained through their local proliferation and BM-independent renewal after birth (Belz and Nutt, 2012; Schulz et al., 2012).

1.2. Transcriptional regulators of B lymphoid and myeloid development

In the hematopoietic system cell fate decisions are controlled by extrinsic and intrinsic factors. The extrinsic factors include cytokines, hormones and direct cell-cell interactions, however, they can have both permissive and instructive roles for hematopoietic cell differentiation (Laiosa et al., 2006a; Rieger et al., 2009). The hematopoietic lineage specification (induction of a lineage specific expression program) and commitment (cell fate determination through repression of alternative gene expression programs) crucially depend on intrinsic transcription factors that set lineage specific networks in the different hematopoietic cells (Laiosa et al., 2006a; Nutt and Kee, 2007). Every lineage is defined by dosage, combination and cross interaction of transcription factors which specifically synergize, antagonize and regulate each other, maintaining transcription factor networks and lineage specific gene expression profiles (Laiosa et al., 2006a). Furthermore, lineage-specific transcription factors determine and maintain the cell fate program through recruitment of proteins and protein complexes that mediate epigenetic gene expression regulation, including DNA methylation, post-translational modifications (PTMs) of histones and nucleosome restructuring (Broske et al., 2009; Gao et al., 2009; Ramirez et al., 2010). The irrefutable evidence for the lineage instructive capacity of transcription factors is the demonstration that, when ectopically expressed, they reprogram a committed cell into another lineage through disruption of the established transcription factor network and creation of a new one (Cobaleda

and Busslinger, 2008). The overexpressed transcription factors activate their endogenous partners, set up autoregulatory loops and disrupt autoregulation of antagonistic factors (Laiosa et al., 2006a). Here, we will focus in more details only on the transcription regulation of the B cell- and the myeloid lineage without the megakaryocyte/erythrocyte branch.

1.2.1. B cell lineage transcription regulators

Conventional B cell (B-2 cell) differentiation proceeds from HSCs through B cell progenitors till the production of surface IgM^+ immature B cells via a series of distinct intermediate steps (Hardy et al., 2007; Medvedovic et al., 2011). Genetic ablation studies have demonstrated that the transcription factors PU.1, Ikaros, E2A, EBF1, and Pax5 play key roles in B cell lineage differentiation (Mandel and Grosschedl, 2010; Nutt and Kee, 2007; Ramirez et al., 2010). The formation of more primitive lymphoid progenitors is dependent on the action of PU.1 and Ikaros and their deficiency severely impairs the early lymphoid lineage development (Nutt and Kee, 2007). Prior to the differentiation of CLPs, Ikaros and PU.1 induce the expression of components of signaling pathways essential for B cell progenitors generation, including interleukin 7 receptor a chain (IL-7Ra) and fms-like tyrosine kinase 3 receptor (Flt-3) (Laiosa et al., 2006a; Mandel and Grosschedl, 2010; Yoshida et al., 2006). Beyond the CLP stage the key determinants of B lymphoid lineage are the transcription factors E2A, EBF1 and Pax5 which function in a hierarchical and combinatorial mode to activate and maintain the B cell-specific gene expression program. The E box-binding protein 2A (E2A) and the early B cell factor EBF1 are upstream of Pax5 in the genetic hierarchy of early B cell development and they are responsible for B cell lineage specification through activation of the expression of B lymphoid genes in pre-pro B cells (Lin et al., 2010; Treiber et al., 2010). Several lines of evidence suggest that E2A acts upstream of EBF1 and it has been shown that *E2A* deficient B lymphoid progenitors are blocked at the transition from ALP to BLP stage of B cell differentiation and fail to express EBF1 and Pax5, whereas *Ebf1*$^{-/-}$ pre-pro B cells express E2A but lack Pax5 (Decker et al., 2009; Inlay et al., 2009; Nutt and Kee, 2007). *E2A* deficient B lymphoid progenitor cells fail to upregulate the early B cell genes *Igll1* (*λ5*) and *Cd79a* and do not rearrange IgH locus beyond D_H-J_H rearrangements (Ikawa et al., 2004). However, EBF1 can also bind *E2A* gene and

conditional inactivation of *Ebf1* leads to downregulation of E2A expression (Treiber et al., 2010). Moreover, E2A binding sites are present on more than two thirds of EBF1 target genes, indicating that both proteins cross regulate each other and function cooperatively in B lymphocyte specification (Lin et al., 2010).

Experiments comparing EBF1 gain- and loss- of function with genome wide analyses of EBF1 binding have identified that the direct EBF1 regulated genes are enriched in components of pre-BCR and B cell receptor (BCR) signaling pathway (Treiber et al., 2010). Targeted inactivation of *Ebf1* results in failure to express early B cell gene components of pre-BCR and BCR, including *Cd79a*, *Cd79b*, *Igll1* (*λ5*), and *Vpreb1*, lack of V_H-DJ_H IgH rearrangements and blockage of B cell development at $B220^+$ $CD19^-$ uncommitted pre-pro B cell stage (Decker et al., 2009; Pongubala et al., 2008). Down in the transcription factor hierarchy, EBF1 initiates chromatin remodeling at the *Pax5* promoter and activates the expression of its direct target gene *Pax5* at the onset of pro B cell stage (Decker et al., 2009; Treiber et al., 2010). Moreover, coordinated E2A and EBF1 binding to *Pax5* gene regulatory elements has been shown (Lin et al., 2010). However, Pax5 is also able to activate *Ebf1* expression, as well as EBF1 binds its own promoter, suggesting that the transcriptional network controlling B cell specification and commitment is not a simple linear cascade but involves multiple combinatorial inputs and cross-regulatory loops (McManus et al., 2011; Nutt and Kee, 2007; Schebesta et al., 2007).

The transcription factor paired box protein 5 (Pax5) is regarded as the master regulator of B cell commitment and a guardian of B cell identity and is expressed throughout B cells lineage from pro B to mature B cells and downregulated in plasma cells (Decker et al., 2009; Medvedovic et al., 2011). Pax5 expression is continuously required throughout B cell development, as supported by the observation that conditional *Pax5* inactivation in mature B cells induces their conversion to functional T cells (Cobaleda et al., 2007a). Pax5 promotes B cell commitment at the transition to the pro B cell stage through repression of B lineage inappropriate genes like crucial receptors required for multipotency (Flt-3), macrophage (M-CSFR) and T cell (Notch1) differentiation, and activation of B cell specific genes (Delogu et al., 2006; Holmes et al., 2006; Nutt et al., 1999; Schebesta et al., 2007). Among Pax5 activated genes are multiple transcription factors (including *Ebf1*) and proteins involved in B cell signaling, adhesion, migration and immune function (e.g. the B cell co-receptor molecule CD19 whose expression marks all cells committed to the B cell lineage)

(McManus et al., 2011; Nutt and Kee, 2007; Schebesta et al., 2007). Pax5 directly binds to promoters and enhancers of its target genes and regulates their expression through induction of active chromatin at the activated genes and removal of active chromatin marks at repressed genes via recruitment of BAF chromatin-remodeling complexes, histone-modifiers and basal transcription factor TFIID complexes. Therefore, Pax5 orchestrates the epigenetic and transcriptional control of its target genes (McManus et al., 2011; Schebesta et al., 2007). EBF1 might also contribute to B cell commitment not only through activation of *Pax5* expression but also through antagonizing the expression of myeloid determinants like *C/EBPα* and *PU.1* in MPP and repression of T cell specific genes in B cell progenitors (Pongubala et al., 2008; Treiber et al., 2010). Interestingly, about 10% of EBF1 bound genome sites have also an additional Pax5 binding site and there is a remarkably high number of Pax5 activated genes and to a lower extend Pax5 repressed genes which have EBF1 binding sites, suggesting the cooperative function of the two factors (Treiber et al., 2010). Additionally to gene expression regulation, Pax5 regulates V_H-DJ_H recombination of the IgH locus by inducing its contraction by looping and its deficiency blocks B cell differentiation at the pro B cell stage shortly after completion of IgH D_H-J_H gene segments rearrangements (Medvedovic et al., 2011). Interestingly, related to the capacity of the three determinants of B lymphoid lineage E2A, EBF1 and Pax5 to repress alternatively lineage choices, it has been shown that the deficiency of any of them permits multilineage differentiation potential (Ikawa et al., 2004; Nutt et al., 1999; Pongubala et al., 2008).

Although the majority of postnatal B lymphocytes are conventional B-2 B cells, an alternative B cell development pathway has been identified and these B-1 cells are preferentially generated during fetal hematopoiesis, while they represent only a minor fraction in adult mice (Barber et al., 2011; Hardy et al., 2007). Interestingly, compared to B-2 cells, the B-1 cells express lower levels of PU.1 and conditional ablation of PU.1 in $CD19^+$ B cells *in vivo*, leads to de-differentiation of B-2 B cell into B-1 resembling cells (Ye et al., 2005).

1.2.2. Transcription factor regulation of the myeloid cell differentiation

The master regulators of the myeloid differentiation are PU.1 and C/EBPs and their interplay is critical for the cell fate decisions in the myeloid lineage (Rosenbauer and

Tenen, 2007). PU.1 is required for development of CMPs and CLPs but not MEPs from HSCs and deletion of PU.1 at the CMP or GMP stage blocks their maturation to granulocytes and monocytes (Iwasaki et al., 2005). PU.1 regulates numerous genes within the myeloid and lymphoid lineages, including those encoding the developmentally important cytokine receptors, macrophage colony stimulating factor receptor (M-CSFR), granulocyte-macrophage colony stimulating factor receptor (GM-CSFR), IL-7Ra and Flt-3 (Carotta et al., 2010; Dakic et al., 2007). The fundamental role of PU.1 as a transcriptional master regulator of myeloid and lymphoid cell fates is highlighted by the presence of PU.1 binding motifs in the regulatory sequences of many macrophage and B cell specific genes and these sites are colocalized by C/EBPs or E2A and EBF1 in the respective lineage (Heinz et al., 2010). In peritoneal macrophages one third of the genome sites occupied by C/EBPα or C/EBPβ are located within 100bp of a PU.1 bound site, suggesting the collaborative function of these transcription factors for the establishment of macrophage gene expression program (Heinz et al., 2010). Furthermore, there are two auto-regulatory loops which activate the *PU.1* locus in a cell type specific manner in macrophages and B cells. Although cis- upstream regulatory element (URE) at -14/-15 kb is essential for *PU.1* expression in all hematopoietic lineages, additional myeloid-specific cis-regulatory element at -12 kb is required to drive high *PU.1* expression in macrophages, and binding of myeloid specific transcription factors like C/EBPα at URE initiates chromatin opening at -12 kb enhancer to drive cell type specific *PU.1* expression (Leddin et al., 2011).

The regulation of hematopoietic differentiation by PU.1 is however not a simple mechanism dependent on presence or absence of expression but it also dependents on the expression level (Mak et al., 2011). It has been shown that low dosage of PU.1 protein induces B cell development, whereas high expression level promotes macrophage differentiation (DeKoter and Singh, 2000). Ectopic expression of PU.1 in *PU.1*$^{+/-}$ fetal liver progenitors cultured on S17 feeder cells in the presence of IL-7 blocks B cell development and induces macrophage differentiation (DeKoter and Singh, 2000). Moreover, in the B cell lineage higher expression of PU.1 is necessary for conventional B-2 cell differentiation, whereas B-1 cells express lower level of PU.1 (Ye et al., 2005). A similar dose effect exists in the myeloid lineages where C/EBPα and high PU.1 levels support monopoiesis, whereas combination of C/EBPα and low PU.1 levels supports granulopoiesis (Yeamans et al., 2007).

Although experiments have shown that PU.1 acts at an earlier stage of the hematopoietic lineage determination compared to C/EBPα (PU.1 is essential for CMP generation, whereas C/EBPα is important for GMP generation (Zhang et al., 2004)), it has been shown that C/EBPα binds and activates the *PU.1* promoter and distal enhancer during myelopoiesis (Kummalue and Friedman, 2003; Yeamans et al., 2007). C/EBPα induces monocytic differentiation when expressed in wild type (WT) murine BM myeloid progenitors but stimulates granulocytic development in *PU.1*$^{kd/kd}$ cells (lacking the *PU.1* distal enhancer URE and expressing 20% of normal PU.1 levels) and induces neither monocytic nor granulocytic differentiation in *PU.1*$^{-/-}$ cells (Yeamans et al., 2007). However, PU.1 and C/EBPs might antagonize each other during DC differentiation, as it has been shown that expression of PU.1 or a dominant negative C/EBP in human CD34^{+} cells enhances Langerhans cell differentiation, whereas C/EBPα and β promote preferentially granulocytic and to some extend monocyte/macrophage differentiation and disrupt DC differentiation induced by PU.1 (Iwama et al., 2002). Furthermore, it has been shown that moderate expression levels of PU.1 and co-expression of the basic leucine zipper (bZip) factor MafB favors macrophage fate, whereas high PU.1 levels induce DC differentiation and repress macrophage differentiation through inhibition of *Mafb* expression and transcriptional activity (Bakri et al., 2005).

Conditional deletion of *PU.1* in distinct hematopoietic progenitor populations leads to blockage of Flt-3 and GM-CSF induced DC differentiation and impairs generation of both cDCs and pDCs, suggesting a crucial role of PU.1 for the development of all DC types (Carotta et al., 2010). However, the differentiation of the variety of DC subsets requires a distinct combination of many different transcription regulators including IRF8, IRF4, BATF3, E2-2, RELB, STAT3, STAT5 and others (Belz and Nutt, 2012). In immature DCs PU.1 and its binding partner interferon-regulatory factor 8 (IRF8) are required for the expression of MHC Class II Transactivator (CIITA), a transcriptional co-activator which is a master regulator of MHC-II gene expression (Smith et al., 2011). Interestingly, IRF8 is expressed not only in DCs, but also in B cells and macrophages, however not in granulocytes and is considered as a determinant of monocyte/macrophage and DC over granulocytic differentiation (Becker et al., 2012; Rosenbauer and Tenen, 2007). Myeloid progenitor cells established from *Irf8*$^{-/-}$ mice differentiate into granulocytes in granulocyte colony stimulating factor (G-CSF) supplemented culture medium. However, retroviral

transduction of *Irf8* results in their differentiation into mature phagocytic macrophages and blocks granulocytic differentiation upon G-CSF treatment (Tamura et al., 2000). In the B cell lineage *Irf8* is directly regulated by Pax5, however on the other hand, IRF8 and PU.1 contribute to the maintenance of *Pax5* expression during early and late B cell development (Decker et al., 2009; Ramirez et al., 2010). Furthermore, *Irf8* deficient mice have a significantly reduced number of pre-pro B cells and increased number of myeloid cells and *Irf8*$^{-/-}$ B cell progenitors show deregulated expression of IRF8 target genes *PU.1* and *Ebf1* (*PU.1* is upregulated and *Ebf1* is downregulated in *Irf8* deficient compared to WT B cell progenitors) (Wang et al., 2008).

C/EBPα deficient mice completely lack GMPs, neutrophil and eosinophil granulocytes, however C/EBPα is not strictly required for granulopoiesis (the formation of granulocytes within the BM) beyond the GMP stage, as its conditional deletion in GMPs does not disrupt normal granulopoiesis *in vitro* (Zhang et al., 1997; Zhang et al., 2004). It has been shown that other factors, like growth-factor independent 1 (GFI1) and C/EBPε are essential for terminal neutrophil granulocytic differentiation (Rosenbauer and Tenen, 2007). Interestingly, C/EBPε is expressed as four isoforms and each of them may display different roles during granulocytic differentiation. Transduction of human CD34^{+} progenitor cells with these isoforms has revealed that C/EBPε$^{32/30}$ isoforms induce eosinophil differentiation; C/EBPε^{27} and C/EBPε^{14} isoform strongly inhibit eosinophil differentiation and gene expression, while C/EBPε^{27} promotes neutrophil/macrophage differentiation and C/EBPε^{14} repressor isoform promotes erythroid differentiation (Bedi et al., 2009). Interestingly, the order of expression of C/EBPα and GATA-2 can differentially control lineage commitment from GMPs to eosinophils, basophils, neutrophils and monocytes (Iwasaki et al., 2006). The last steps of neutrophil terminal differentiation are characterized by increased expression levels of PU.1 and the C/EBP protein family members C/EBPβ, C/EBPδ and C/EBPζ (Bjerregaard et al., 2003; Borregaard, 2010). Furthermore, C/EBPβ can substitute for C/EBPα during emergency granulopoiesis or when expressed from its endogenous locus (Hirai et al., 2006; Jones et al., 2002). A detailed description of C/EBPβ functions during hematopoiesis will be given in the next subchapter.

1.2.3. C/EBPβ structure, regulation and function in the hematopoietic system

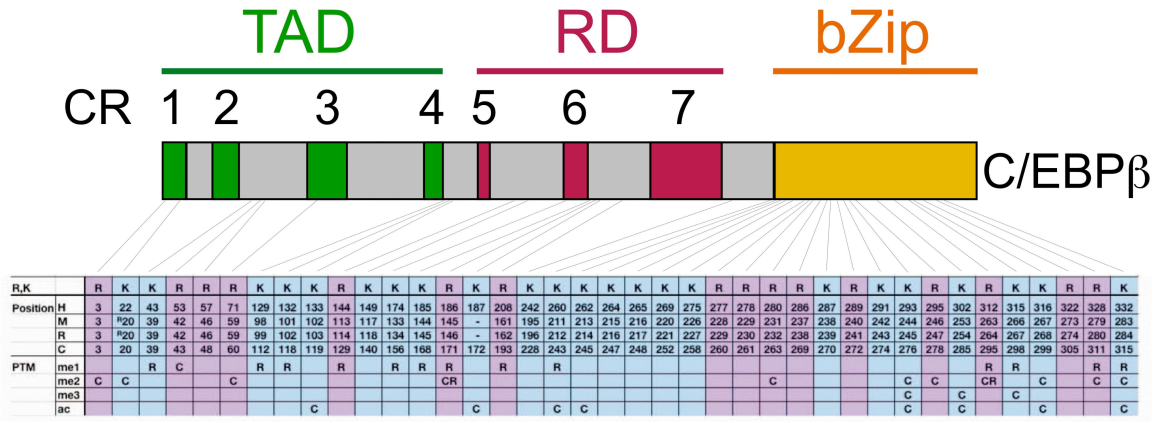
CCAAT enhancer binding protein (C/EBP) family consists of six members: C/EBPα, C/EBPβ, C/EBPδ, C/EBPε, C/EBPγ, and C/EBPζ. C/EBPs regulate cell proliferation, differentiation, metabolism, inflammation, apoptosis and tumorigenesis in a variety of cell types, including hepatocytes, adipocytes and hematopoietic cells (Akagi et al., 2008; Cao et al., 1991; Chen et al., 1997; Johnson, 2005; Ramji and Foka, 2002; Smink and Leutz, 2012; Tanaka et al., 1995; Wethmar et al., 2010; Zahnow, 2009). C/EBPs orchestrate cell type specification in combination with other transcription factors. For example, C/EBPα and β require peroxisome proliferator-activated receptor gamma (PPARγ) for the induction of white adipogenic differentiation from fibroblasts (Rosen et al., 2002; Zuo et al., 2006), while co-expression of C/EBPβ and PRDM16 in fibroblasts or myoblastic precursors induces brown adipogenic differentiation (Kajimura et al., 2009). C/EBPβ cooperates with c-Myb in the activation of myeloid specific genes in heterologous cell types (Ness et al., 1993), and, in combination with PU.1, C/EBPα and β may evoke myeloid trans-differentiation of fibroblasts (Feng et al., 2008).

As a member of C/EBP transcription factors family, C/EBPβ protein contains highly conserved C-terminal DNA binding and leucine zipper dimerization domain (bZip), central regulatory domain (RD) and N-terminal transactivation domain (TAD). The TAD and the RD consist of several conserved regions (CRs) with modular functions which are highly conserved between orthologous proteins and paralogous members of vertebrate C/EBPs (Kowenz-Leutz et al., 1994; Leutz et al., 2011). Individual CR modules are separated by low complexity regions (LCRs) which differ between family members and are polymorphic within the same family. Deletion and functional analyses suggest that LCRs may function as flexible hinges and recipients of signal dependent PTMs while CRs may serve scaffolding functions for regulated intramolecular interactions and docking of chromatin modulating complexes and the transcription machinery (Leutz et al., 2011). The N terminus of C/EBPβ is involved in the transcription activation and it is shown to interact with mediator complexes, basal transcriptional machinery (TBP/TFIIB) and with transcription co-factors, including chromatin-remodeling complexes (SWI/SNF), histone acetyltransferase (p300), methyltransferases (PRMT4 and G9a) and several other chromatin regulatory complex components (Kowenz-Leutz and Leutz, 1999; Kowenz-Leutz et al., 2010;

Lee et al., 2010a; Mink et al., 1997; Mo et al., 2004; Nerlov and Ziff, 1995; Pless et al., 2008; Steinberg et al., 2012). C/EBP proteins are active as dimers and via its leucine zipper dimerization domain C/EBPβ can form homo- or heterodimers with other C/EBPs or other bZip proteins. Additionally, C/EBPβ dimers may interact through the C-terminal bZip domain with other transcription factors, like c-Myb and PU.1 and this interaction mediates their cooperative target gene activation (Miller, 2009; Tsukada et al., 2011). Furthermore, C/EBPβ binding sequences are found in the regulatory regions of many genes involved in the acute-phase response, inflammation, and hematopoietic differentiation including C reactive protein, α1-acid glycoprotein, α2-macroglobuin, hemopexin, haptoglobin; genes coding for the cytokines TNF-α, IL-1β, IL-6, IL-8, IL-12, G-CSF, CCL3 (also known as macrophage inflammatory protein-1α, MIP-1α), CCL4 (MIP-1β); and cytokine receptors G-CSFR, GM-CSFR and M-CSFR (Tsukada et al., 2011).

C/EBPβ is expressed in three protein isoforms - long ones (LAP* and LAP, liver activating protein) with TAD and truncated form (LIP, liver inhibitory protein), which acts as a dominant inhibitory peptide lacking the N-terminal activation function (Descombes and Schibler, 1991). The three C/EBPβ isoforms are generated through a process of alternative translation initiation which is regulated by a highly conserved out of frame small upstream open reading frame (uORF) and the translation initiation is under the control of the mammalian target of rapamycin (mTOR). Regulation of the isoform ratio is crucial for the maintenance of the balance between cell proliferation and differentiation and its dysregulation is a prerequisite for a tumorigenesis (Calkhoven et al., 2000; Wethmar et al., 2010; Zahnow, 2009). However, the effect of C/EBPβ on the cell proliferation is cell type specific and in some cases C/EBPβ can have growth-promoting and in others growth-arresting activity (Berberich-Siebelt et al., 2006; Chen et al., 1997; Gutsch et al., 2011; Hirai et al., 2006; Sebastian et al., 2005; Tang et al., 2003; Xie et al., 2004). Additionally, PTMs can modulate C/EBPβ transcriptional activity, DNA binding, protein interactions and subcellular localization (Nerlov, 2008; Tsukada et al., 2011; Zahnow, 2009). It has been shown that C/EBPβ functions are regulated by phosphorylation (Kowenz-Leutz et al., 1994; Lee et al., 2010b; Lynch et al., 2011; Mo et al., 2004; Nerlov, 2008), methylation (Kowenz-Leutz et al., 2010; Leutz et al., 2011; Pless et al., 2008), acetylation (Cesena et al., 2007; Cesena et al., 2008) and SUMOylation (SUMO, small ubiquitin-like modifier) (Berberich-Siebelt et al., 2006; Eaton and Sealy, 2003; Leutz et al., 2011) (Fig. 1.2).

Mass spectrometry data have shown that many arginine (R) residues in TAD, RD and bZip of C/EBPβ are mono- or dimethylated and individual or combined mutation of these residues to alanine (A) results in increased reporter and endogenous gene activation potential, demonstrating that the PTM pattern regulates C/EBPβ functionality (Leutz et al., 2011). The complexity of C/EBPβ structure thus suggests highly modular, context specific N-terminal functions and a flexible adaptation to signaling and metabolic events (Leutz et al., 2011; Nerlov, 2008).



R,K		R	K	K	R	R	R	K	K	K	R	K	K	K	R	K	R	K	K	K	K	K	K	R	R	R	R	K	R	K	K	R	R	K
Position	H	3	22	43	53	57	71	129	132	133	144	149	174	185	186	187	208	242	260	262	264	265	269	275	277	278	280	286	287	289	291	293	295	302	312	315	316	322	328	332
	M	3	R20	39	42	46	59	98	101	102	113	117	133	144	145	-	161	195	211	213	215	216	220	226	228	229	231	237	238	240	242	244	246	253	263	266	267	273	279	283
	R	3	R20	39	42	46	59	99	102	103	114	118	134	145	146	-	162	196	212	214	216	217	221	227	229	230	232	238	239	241	243	245	247	254	264	267	268	274	280	284
	C	3	20	39	43	48	60	112	118	119	129	140	156	168	171	172	193	228	243	245	247	248	252	258	260	261	263	269	270	272	274	276	278	285	295	298	299	305	311	315
PTM	me1			R	C			R	R		R		R	R	R		R		R															R	R			R	R
	me2	C	C				C								CR												C					C	C		CR		C		C	C
	me3																															C		C		C				
	ac									C						C			C	C												C		C			C			C

Fig. 1.2 C/EBPβ is extensively post-translationally modified by methylation and acetylation. Localization of evolutionary conserved arginine (R) or lysine (K) residues in C/EBPβ LAP* in relation to the protein structure. Underneath: mass spectrometry analyses have identified mono-(me1), di-(me2), tri-(me3) methylation, and acetylation (ac) on the respective residue in rat (R) and chicken (C) C/EBPb. TAD – transactivation domain, RD - regulatory domain, bZip - basic DNA binding and leucine zipper dimerization domain, CR - conserved region, H - human, M - mouse, PTM - post-translational modifications. Re-drawn from (Leutz et al., 2011).

C/EBPβ deficient mice display defects in both lymphoid and myeloid lineages in the hematopoietic system. These mice have decreased number of BM B lymphocytes, which additionally display impaired expansion in long-term culture (Chen et al., 1997). With aging, *C/EBPβ* deficient mice develop lymphoproliferative and myeloproliferative alterations (Screpanti et al., 1995). In the myeloid lineage, C/EBPβ is essential for macrophage bactericidal and tumor cytotoxicity role (Tanaka et al., 1995) and is crucial for the survival of transformed macrophages through induction of the autocrine-acting pro-survival factor insulin-like growth factor I (IGF-I) (Wessells et al., 2004). C/EBPβ is important for the “emergency” granulopoiesis, as *C/EBPβ* deficient hematopoietic progenitor cells have impaired response to GM-CSF and G-

CSF and *C/EBPβ* deficient neutrophils exhibit increased apoptosis (Akagi et al., 2008; Hirai et al., 2006). Furthermore, *C/EBPβ* knock-in mice have been generated in order to examine the function of a single C/EBPβ isoform. In mice lacking C/EBPβ LAP isoform, the induction of C/EBPβ target genes in activated macrophages is abolished similarly to *C/EBPβ* deficiency, however no impairment of intracellular bacteria killing is observed, suggesting that the extended long isoform LAP* could compensate for some, but not for all functions of the LAP isoform (Uematsu et al., 2007). Furthermore, after partial hepatectomy *C/EBPβ*$^{\Delta uORF}$ hepatocytes (lacking only C/EBPβ LIP expression) demonstrate decreased proliferation, delayed cell cycle entry and constant repression of E2F regulated genes (Wethmar et al., 2010). Studies with *C/EBPβ*$^{-/-}$, *C/EBPβ*^{LIP} and *C/EBPβ*$^{\Delta uORF}$ mice have shown opposing roles of long and short C/EBPβ isoforms for the regulation of the osteoclast differentiation, demonstrating that lack of LIP leads to impairment of osteoclast differentiation, whereas *C/EBPβ*$^{-/-}$ and *C/EBPβ*^{LIP} genotypes (lacking the long C/EBPβ isoforms) exhibit strongly enhanced differentiation of the bone-resorbing cells (Smink et al., 2009; Wethmar et al., 2010). Furthermore, studies with mice carrying a targeted deletion of two CREB-binding sites in the *C/EBPβ* promoter (*βΔCre* mice) and incapable of upregulation of *C/EBPβ* upon inflammatory stimuli have shown that high C/EBPβ level is crucial for the induction of anti-inflammatory macrophage genes (Ruffell et al., 2009).

1.3. Cell plasticity and cell reprogramming

Until recently cellular and developmental biology have postulated a dogma that lineage commitment, established through the collaborative action of transcription factors and epigenetic regulators, is irreversible (Carotta and Nutt, 2008). However, studies of the last two decades have provided evidence that committed cells do not lose their developmental potential and may retain lineage promiscuity. Lineage infidelity can be observed, for example, in classical Hodgkin lymphoma where the B cell specification transcription factor E2A is expressed, however due to an overexpression of two E2A antagonists (activated B cell factor 1 (ABF-1) and inhibitor of differentiation 2 (Id2)) the expression of B cell specific genes is lost, while B cell lineage inappropriate genes are expressed (Janz et al., 2006; Mathas et al., 2006). Furthermore, bi-phenotypic acute leukemia with expression of both, myeloid and

lymphoid cell surface markers have been described (Matutes et al., 1997), and lineage switching of B cell lymphoma, acute lymphoblastic leukemia or bi-phenotypic leukemia to acute myeloid leukemia (Muroi et al., 1995; Slany, 2009; Tsuboi et al., 2002), as well as trans-differentiation of follicular lymphoma to histiocytic/DC sarcomas with clonal immunoglobulin rearrangements have been reported (Feldman et al., 2008), suggesting the potential role of cell reprogramming during leukemogenesis. However, lineage plasticity might be a characteristic not only of transformed cells but also of normal cells. For example, HSCs and progenitor cells express genes typical for different lineages and this process, known as lineage priming, might be the reason for the broad developmental plasticity of progenitor cells (Cobaleda and Busslinger, 2008; Laiosa et al., 2006a). Furthermore, mRNA for M-CSFR and other macrophage specific genes are expressed in granulocytes and they can be trans-differentiated into macrophage-like cells upon M-CSF stimulation (Sasmono et al., 2007). The gene expression profile of preadipocytes is much closer to that of macrophages than to adipocytes and preadipocytes could efficiently be converted into Mac-1^+ F4/80^+ macrophages after *in vivo* transplantation into the mouse peritoneal cavity (Charriere et al., 2003).

1.3.1. Hematopoietic cell reprogramming

Aside from the lineage infidelity seen in tumor cells and the physiological cellular plasticity described above, in the recent years experimental hematopoietic cell reprogramming has been achieved by stimulation through exogenously expressed cytokine receptors or targeted inactivation or enforced expression of specific transcription factors. For example, ectopical expression of M-CSFR in murine pre B cells reprograms them to phagocytic macrophages with rearranged immunoglobulin gene loci (Borzillo et al., 1990). Likewise, exogenous expression of IL-2Rβ and GM-CSFR in CLP and pro T cells reveals their latent myeloid differentiation potential (Cobaleda and Busslinger, 2008; Hsu et al., 2006; Iwasaki-Arai et al., 2003; King et al., 2002; Kondo et al., 2000). At the molecular level, ectopical IL-2Rβ signaling leads to upregulation of the cytokine receptors GM-CSFR and M-CSFR as well as the key myeloid transcription factor C/EBPα (Hsu et al., 2006; Kondo et al., 2000). Enforced expression of Flt-3 in MEPs, which normally do not express Flt-3, results in the acquisition of pDC and cDC potential (Onai et al., 2006).

A particularly dramatic example of lineage plasticity resulting from inactivation of a transcription factor comes from studies of the deficiency of the B cell commitment factor Pax5. It has been shown that *Pax5*$^{-/-}$ pro B cells are no longer B cell lineage restricted but entail the capacity to de-differentiate into macrophages, osteoclasts, granulocytes, NK cells, DCs and T cells (Carotta and Nutt, 2008; Cobaleda et al., 2007b; Nutt et al., 1999) (Fig. 1.3). At the molecular level, *Pax5* deficiency allows promiscuous expression of myeloid, erythroid and T cell genes in *Pax5*$^{-/-}$ pro B cells, indicating the essential function of Pax5 to repress non B lymphoid genes (Delogu et al., 2006; Nutt et al., 1999). Furthermore, conditional inactivation of *Pax5* in mature B cells results in their de-differentiation to a progenitor state with a multilineage potential, underlining the continuous requirement of Pax5 to maintain B cell lineage commitment (Cobaleda et al., 2007a). Interestingly, *Cd19* locus insertion of the gene coding for osteoclastogenesis promoting chemokine *Ccl3* (a gene repressed by Pax5) and its ectopic expression in B cells results in enhanced osteoclast formation and bone loss (Delogu et al., 2006).

A similar capacity for multilineage differentiation has been reported for *E2A* deficient hematopoietic progenitor cells. These cells co-express genes associated with other hematopoietic lineages, such as *Gata3* and *Tcf7* for T cells, *Gata1* and *Epor* for erythroid cells, as well as *G-CSFR* and *M-CSFR* for myeloid cells and can *in vivo* differentiate into T cells, NK cells, macrophages, granulocytes, erythrocytes and DCs but not to mature B cells (Ikawa et al., 2004). Likewise, it has been shown that *Ebf1* deficient B220^{+} CD19^{-} B lymphoid progenitor cells have the potential to reconstitute several alternative hematopoietic lineages. These *Ebf1*$^{-/-}$ cells can *in vivo* generate Mac-1^{+} Gr-1^{+} myeloid cells, CD11c^{+} DCs, DX5^{+} NK cells, CD4^{+}CD8^{+} double-positive and single-positive thymocytes, however have a neglectable erythroid potential (Pongubala et al., 2008).

Furthermore, *PU.1* excision in CD19^{+} B cells results in a B-2 to B-1 cell reprogramming (Ye et al., 2005). Knock-down of MTA3, a cell type specific component of Mi-2/NuRD co-repressor complex, impairs BCL-6-dependent repression of plasma cell differentiation program and activates this program in B lymphocytes. Conversely, ectopical co-expression of BCL-6 and MTA3 in plasma cells leads to their reprogramming to B lymphocytes through repression of plasma cell fate specific transcripts, including the master regulator of plasma cell identity Blimp-1, and reactivation of the B cell transcription pattern (Fujita et al., 2004).

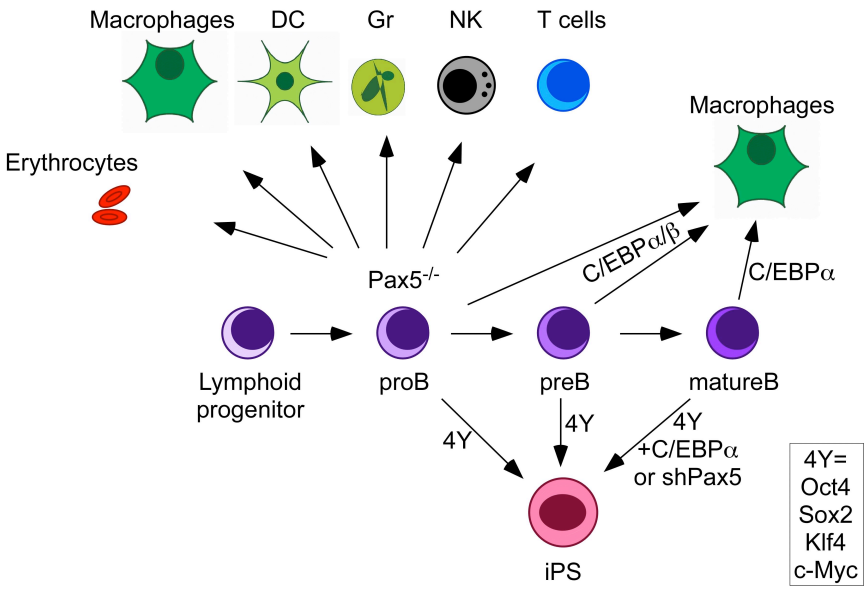


Fig. 1.3 B lymphoid plasticity.

In the absence of B cell lineage commitment factor Pax5, B cell development is blocked at the pro B cell stage. These cells are not committed to the B cell lineage but are able to differentiate into other hematopoietic cell types. It has been also shown that forced expression of C/EBPα or C/EBPβ in B cell progenitors or mature splenic B cells induces their trans-differentiation into macrophages. Furthermore, experiments have demonstrated that Oct4, Sox2, Klf4, and c-Myc (4 Yamanaka transcription factors, 4Y) induce iPS cell formation from pro B and pre B cells, however these factors are ineffective in mature B cells, which require additional C/EBPα overexpression or Pax5 knock down. DC - dendritic cells, Gr - granulocytes, NK - natural killers. Figure is adapted from (Carotta and Nutt, 2008; Graf and Busslinger, 2008; Laiosa et al., 2006a).

As already mentioned, not only targeted gene inactivation but also ectopical expression of transcription factors that play a central role in the physiological cell differentiation can lead to disruption of the established cell fate. It has been shown that B lymphoid cells can be reprogrammed to macrophages by the ectopic expression of C/EBPα and β transcription factors (Bussmann et al., 2009; Xie et al., 2004) (Fig. 1.3.), even though other groups have reported that this could be obtained only in *Pax5*$^{-/-}$ B cells, most probably due to accomplishment of low C/EBP expression or because of the lymphoid growth conditions (Heavey et al., 2003). This reprogramming process is direct and does not involve retro-differentiation into early hematopoietic progenitors or stem cells (Di Tullio et al., 2011). Further characterization of the B cell to macrophage conversion has demonstrated that it does not proceed with significant DNA methylation changes but is accompanied only by histone modification alterations on key lineage specific genes (Rodriguez-Ubreva

et al., 2012). Recently it has been also shown that cell division is not obligatory for C/EBPα-induced B-to-macrophage reprogramming (Di Tullio and Graf, 2012). Similar to B cells, MEPs and CLPs could also be de-differentiated to functional macrophages by enforced C/EBPα expression (Fukuchi et al., 2006; Hsu et al., 2006). The lymphoid to myeloid conversion by C/EBPs is dependent on both synergy with the endogenous PU.1 and repression of B lineage commitment factor Pax5 (Bussmann et al., 2009; Hsu et al., 2006; Xie et al., 2004). This repression of Pax5 functions is due to inhibition of the protein activity (Xie et al., 2004) and not due to direct binding of C/EBPβ to *Pax5* promoter (Pal et al., 2009). On the other hand, enforced expression of EBF1 in CMPs or GMPs downregulates the expression of C/EBPα and PU.1 and induces reprogramming into $CD19^+$ cells (Pongubala et al., 2008). Similarly, C/EBPα-induced B cell to macrophage reprogramming could be delayed by Pax5 coexpression, whereas EBF1 has weaker effect and E2A shows no influence (Bussmann et al., 2009). In the same set of experiments, lack of endogenous PU.1 inhibits CD11b upregulation without affecting CD19 downregulation (Bussmann et al., 2009; Xie et al., 2004). Even fibroblasts, not related to the hematopoietic lineage cell type, could be reprogrammed to macrophage-like cells by the myeloid determinant factors CEBPα and C/EBPβ in combination with PU.1 (Feng et al., 2008). The overexpression of another transcription factor Oct4 in combination with SCF and Flt-3L cytokines supplemented media reprograms human fibroblasts directly without involvement of a pluripotent state into $CD45^+$ multipotent hematopoietic progenitor cells which can be further differentiated into granulocytes, monocytes, erythrocytes and megakaryocytes after additional cytokine stimulation *in vitro* or after transplantation *in vivo* (Szabo et al., 2010).

Unlike synergism during B cell or fibroblast to macrophage reprogramming, PU.1 and C/EBPs can also antagonize each other and determine alternative lineage choices. For example, PU.1 and C/EBPα / C/EBPβ have opposite effects on Langerhans cell differentiation (Iwama et al., 2002). Accordingly, C/EBPα and β reprogram committed T cell progenitors to inflammatory macrophages, whereas PU.1 reprograms them under the same culture conditions into myeloid DCs (Laiosa et al., 2006b). GATA-3, a transcription factor which is generally considered to be specific and essential for T cell differentiation, can convert T cell progenitors to mast cells (Taghon et al., 2007). However, Notch signaling can counteract the ability of C/EBPs, PU.1 and GATA-3 to reprogram the T cell progenitors (Laiosa et al., 2006b; Taghon et al., 2007).

Furthermore, similarly to enforced Flt-3 expression, its downstream targets STAT3 or PU.1 instruct MEPs to differentiate into pDC, cDCs and myelomonocytic cells while suppressing megakaryocyte/erythrocyte development (Onai et al., 2006). Expression of constitutively active β-catenin in lymphoid or myeloid hematopoietic progenitors converts them into cells which express lineage inappropriate genes and have multilineage differentiation potential (Baba et al., 2005). Forced expression of GATA-1 in CLPs leads to megakaryocyte/ erythrocyte trans-differentiation and inhibition of the normal lymphoid development (Iwasaki et al., 2003). Interestingly, the reprogramming outcome might also be defined by the sequence of transcription factor ectopical expression. For example, when alternating the order of ectopical expression of C/EBPα and GATA-2, CLPs can be differentially reprogrammed to multiple myeloid cell subtypes including monocytes, neutrophils, eosinophils, basophils and mast cells (Iwasaki et al., 2006).

1.3.2. Cell plasticity of the non-hematopoietic lineages

Numerous studies have shown that non-hematopoietic lineage cells can also be trans-differentiated into another cell type. For example, the exogenous expression of the skeletal myogenesis master regulatory transcription factor MyoD in fibroblasts or other cell types is sufficient to activate skeletal muscle differentiation program (Weintraub et al., 1989). The combination of three transcription factors involved in heart development, Gata4, Mef2c and Tbx5, directly reprograms cardiac or dermal fibroblasts into functional cardiomyocytes *in vitro* and *in vivo* (Ieda et al., 2010). The ectopical expression of the three key developmental regulators Ngn3, Pdx1 and Mafa converts mouse exocrine pancreatic cells into insulin-producing β-cells *in vivo* (Zhou et al., 2008), whereas Ngn3 is sufficient to trans-differentiate hepatic progenitors into β-cell islets (Yechoor et al., 2009). Transduction of Gata4, Hnf1a and Foxa3, and inactivation of $p19^{Arf}$ in mouse fibroblasts directly reprograms these cells into functional hepatocyte-like cells *in vitro* and *in vivo* (Huang et al., 2011). Forced expression of PR domain containing 16 (PRDM16) and C/EBPβ in mouse and human fibroblasts is sufficient to induce a functional brown fat differentiation program *in vitro* and *in vivo* after transplantation into mice (Kajimura et al., 2009). Furthermore, fibroblasts could also be directly reprogrammed into functional neurons by the combinatorial expression of defined neural specific transcription factors, even

in combination with microRNAs (miRNAs) (Ambasudhan et al., 2011; Vierbuchen et al., 2010). Moreover, a cocktail of the four reprogramming factors (Oct4, Sox2, Klf4, and c-Myc) and reprogramming medium supplemented with neural growth factors can efficiently trans-differentiate fibroblasts into expandable neural stem/progenitor cells that have the ability to give rise to functional mature neurons and glial cells (Kim et al., 2011), making direct reprogramming a very attractive method for derivation of expandable patient specific cells of interest.

A groundbreaking discovery which has challenged all concepts about cell differentiation and plasticity is the finding that fibroblasts can be reprogrammed into the so-called iPS (induced pluripotent stem) cells through ectopic expression of the four transcription factors Oct4, Sox2, c-Myc and Klf4. These iPS cells can generate viable chimaeric animals with germline contribution and their similarities and differences from embryonic stem cells are subject to extensive research (Bar-Nur et al., 2011; Polo et al., 2010; Stadtfeld et al., 2010; Takahashi and Yamanaka, 2006; Wernig et al., 2007). On the other hand, Oct4 ectopic expression alone is enough to reprogram mouse and human neuronal stem cells to iPS cells (Kim et al., 2009a; Kim et al., 2009b). Interestingly, hematopoietic cells can also give rise to iPS cells by transduction with the four reprogramming factors, however, mature B cells require additional C/EBPα overexpression or Pax5 knock down (Eminli et al., 2009; Graf, 2009; Hanna et al., 2008; Loh et al., 2009; Loh et al., 2010) (Fig. 3.1).

1.4. Monocyte and macrophage heterogeneity and plasticity

Macrophages and their precursors peripheral blood monocytes are non-homogenous populations (Geissmann et al., 2003; Gordon and Taylor, 2005; Yona and Jung, 2010). There are two main murine monocyte subsets *in vivo* – $CD11b^+$ Ly-6C/Gr-1^+ “inflammatory” monocytes, which are recruited to the sites of peripheral inflammation, and $CD11b^+$ Ly-6C/Gr-1^- “resident” monocytes, which home to non-inflamed tissue. These monocyte subsets share common monocyte characteristics, such as CD11b and M-CSF receptor expression, they are phagocytic and have a potential to differentiate into DCs, however they differ in the expression of adhesion molecules and chemokine receptors such as CD62L (L-selectin), CCR2, CX_3CR1 which defines the distinct mechanisms of their tissue recruitment and biological functions (Arnold et al., 2007; Auffray et al., 2007; Geissmann et al., 2003; Nahrendorf et al., 2007;

Sunderkotter et al., 2004). Furthermore, there is a developmental relationship between the two subpopulations and it has been shown that Ly-6C/Gr-1 expression is downregulated *in vitro* and *in vivo* during monocyte maturation (Arnold et al., 2007; Drutman et al., 2012; Sunderkotter et al., 2004).

Similarly to blood monocytes, tissue macrophages are heterogeneous and that reflects the variety of specific, even opposite functions they have in the different anatomical locations, e.g. pro-inflammatory or anti-inflammatory, digestion of old or damaged tissue or tissue healing, immunogenic or immune-tolerant functions (Arnold et al., 2007; Gordon and Taylor, 2005; Yona and Jung, 2010). Resembling T helper type 1 and type 2 polarization, macrophages could be polarized into classically activated (M1) and alternatively activated (M2) macrophage phenotypes, which have distinct biological functions and different gene expression profiles. Bacterial moieties (e.g. LPS) and IFNγ polarize macrophages toward inflammatory M1 phenotype, whereas M2 anti-inflammatory polarization is induced after stimulation with IL-4, IL-13, immune complexes/TLR ligands, TGFβ or IL-10, and based on these polarizing stimuli M2 macrophages can be further subdivided into additional categories (Cassetta et al., 2011; Solinas et al., 2009). M1 macrophages provide a defense against infections and tumors, produce high levels of inflammatory cytokines, and activate immune response, whereas M2 polarized macrophages promote scavenging of cell debris, angiogenesis, remodeling and tissue repair, display immune-regulatory functions and might promote tumor progression. Classically activated M1 macrophages are characterized by high production of TNF-α, IL-1β, IL-6, IL-12 and IL-23, high amounts of reactive nitrogen and oxygen intermediates and efficient antigen presenting capability. Hallmarks of M2 macrophages are high expression of IL-10, IL-1 receptor antagonist (IL-1RA) and IL-1 decoy receptor and low expression of IL-12, high expression of mannose, scavenger and galactose receptors, predominance of the arginase pathway and poor antigen presenting capability (Biswas and Mantovani, 2010, 2012; Mantovani, 2008; Mantovani et al., 2002; Solinas et al., 2009) (Fig. 1.4). Loss of equilibrium between M1 and M2 cells may lead to pathological outcomes: M1 macrophage predominance might lead to chronic inflammatory diseases, whereas M2 macrophage majority could result in severe immune suppression (Solinas et al., 2009). However, M1 and M2 polarized phenotypes are extremes of a spectrum of macrophage phenotypes and oversimplification of the whole range of intermediate functional states. Furthermore,

in response to environmental signals macrophages can display a considerable plasticity and alter their functional state (Biswas and Mantovani, 2010; Cassetta et al., 2011). Additionally, besides M1 and M2 macrophages, another macrophage polarization phenotype has been identified, namely regulatory macrophages, which might play a crucial role in diminishment of immune responses and restriction of inflammation. Similar to M2 macrophages, these macrophages produce high levels of IL-10, however, unlike M2 cells, they do not participate in the extracellular matrix production and in many cases might express high levels of co-stimulatory molecules (Cassetta et al., 2011; Mosser and Edwards, 2008).

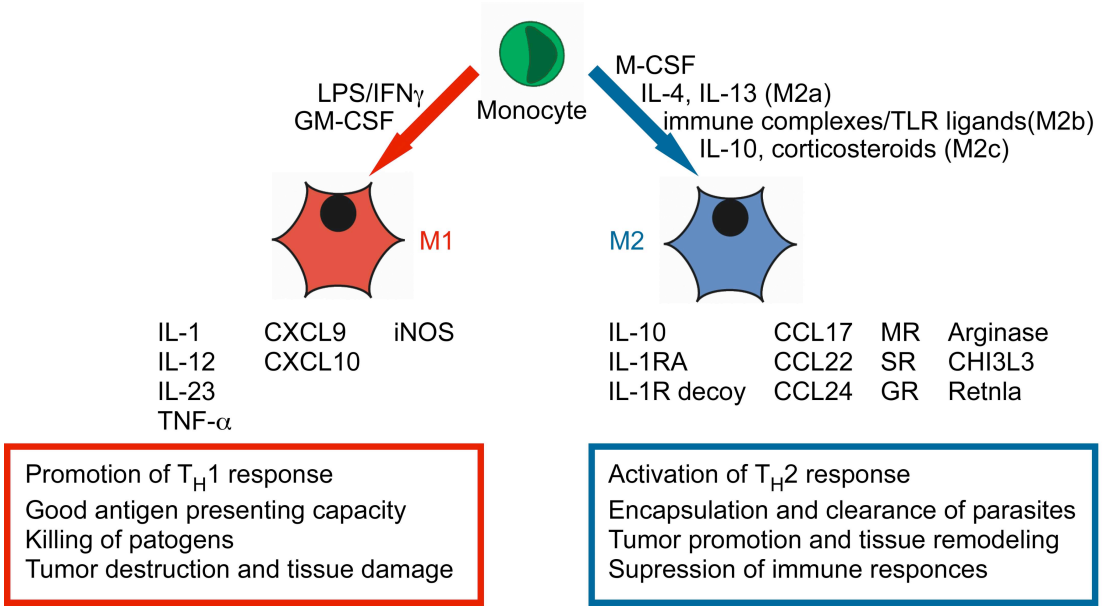


Fig. 1.4 Macrophage polarization.

In the presence of LPS/IFNγ monocytes can be polarized into M1 classically activated macrophages, which produce high levels of IL-12, IL-1, IL-23 and TNF-α, attract T_H1 cells, have high cytotoxic activity against microorganisms and neoplastic cells and have good antigen presenting capacity. On the other hand, stimulation with IL-4, IL-13, IL-10, or corticosteroids results in M2 alternatively macrophage polarization, which promotes angiogenesis, tissue remodeling and repair of damaged tissues and might suppress the inflammatory response by down-regulating M1-mediated functions and adaptive immunity. LPS - lipopolysaccharide, IFNγ - interferon gamma, GM-CSF - granulocyte/macrophage - colony stimulating factor, M-CSF - macrophage - colony stimulating factor, IL - interleukin, IL-1RA - IL-1 receptor antagonist, TNF-α - tumor necrosis factor-α, CXCL - chemokine (C-X-C motif) ligand, iNOS - inducible nitric oxide synthase, CCL - chemokine (C-C motif) ligand, MR - mannose receptor, SR - scavenging receptor, GR - galactose receptor, CHI3L3 - chitinase 3-like 3, Retnla- resistin like alpha, T_H - T helper cells. Figure is adapted from (Biswas and Mantovani, 2010; Solinas et al., 2009).

Interestingly, C/EBPβ regulates various macrophage functions and macrophage-associated genes. It has been shown that C/EBPβ is essential for macrophage bactericidal and tumor cytotoxicity role (Tanaka et al., 1995). *C/EBPβ* deficiency affects many macrophage genes, as these genes are deregulated in *C/EBPβ*$^{-/-}$ macrophages upon IFNγ and/or LPS stimulation: *G-CSF*, *Clec4e*, *Il1b*, *Tnf*, *Il6*, *Nos2*, *Il12a*, *Ptges*, *Cxcl13* gene expression could not be upregulated upon inflammatory stimuli, whereas *Il12b* and *Ccl4* (*Mip1b*) genes have higher expression in *C/EBPβ*$^{-/-}$ macrophages compared to WT (Gorgoni et al., 2002; Matsumoto et al., 1999; Tanaka et al., 1995; Uematsu et al., 2007). Deletion of CREB-binding sites in *C/EBPβ* promoter does not affect the induction of M1 pro-inflammatory genes upon macrophage activation, but impairs the upregulation of M2 anti-inflammatory genes, suggesting that C/EBPβ upregulation in macrophages is important for acquisition of anti-inflammatory gene expression profile (Ruffell et al., 2009). Furthermore, recently it has been shown that C/EBPβ is a crucial regulator of the tumor-induced tolerance and immune suppression and its deficiency results in abrogation of suppressive myeloid cells activity and reduced expression of crucial components of myeloid immuno-inhibitory machinery such as arginase 1 (*Arg1*) and inducible nitric oxide synthase (*Nos2*) (Marigo et al., 2010). Remarkably, tumor-associated macrophages and monocyte-type myeloid-derived suppressor cells in general have a M2 anti-inflammatory phenotype and instead of countracting tumor progression they rather promote it (Biswas and Mantovani, 2010; Solinas et al., 2009). All these data suggest a pivotal role of C/EBPβ in the regulation of the inflammation and immune responses.

1.5 Aims of the thesis

C/EBPβ is a transcription factor which plays a crucial role in many cellular processes in a variety of cell types. Several lines of evidence have indicated that in the hematopoietic system C/EBPβ regulates the proliferation, functionality and development of B cells, monocytes/macrophages, granulocytes and DCs. But how does a single transcription factor fulfill all these tasks? Is the functional diversity orchestrated only by regulation of the isoform expression or is it also dependent on the acquisition of signaling dependent PTM pattern? Furthermore, whether and how the modular structure of C/EBPβ influence cell fate decisions is not known. Therefore, a refined molecular understanding of the mechanism of cell differentiation induced by C/EBPβ was a driving force for the current study. Based on the observation that ectopical C/EBPβ expression in B cell progenitors may reprogram them into myeloid cells, we addressed the following questions:

I. Which C/EBPβ protein modules are important for its reprogramming function?
To answer this question B cell progenitors were substituted with the WT isoforms and functional mutants of C/EBPβ, including conserved region (CR) deletion mutants and post-translational modification (PTM) site mutants.

II. Does the C/EBPβ structure and signaling dependent PTMs navigate B cell reprogramming towards distinct differentiation outcomes?
To gain insight whether structural alterations in C/EBPβ may orchestrate different myeloid lineage differentiation outcomes, I performed detailed analyses of characteristics associated with granulocytic, M1/M2 monocyte/macrophage and DC differentiation.

2. Materials and Methods

2.1. Mouse work and isolation of primary B lineage cells and fetal liver cells

2.1.1 Mouse strains and genotyping

Primary B cell progenitors were isolated from WT C57BL/6, as well as *C/EBPβ*$^{-/-}$ and *Irf8*$^{-/-}$ mice, which were described before (Holtschke et al., 1996; Sterneck et al., 1997). All mice were bred and maintained in the animal facilities of Max Delbrück Center and Charité, Campus Virchow-Klinikum in accordance with guidelines from institutional Animal Care and Use Committee.

For the mouse genotyping, tailtips were lysed overnight in 50 µl Proteinase K buffer (100 mM Tris pH 8.0, 0.2% SDS, 200 mM NaCl, 5 mM EDTA, 30 µg/ml Proteinase K (Sigma)) at 55°C in a horizontal shaker at 550 rpm. The digested tailcuts were incubated at 95°C for 10 min for Proteinase K inactivation and 150 µl distilled water (dwater) was added. For the genotyping PCR reactions, 1 µl of the liquid phase was used as DNA template. The same procedure was performed for the genotyping of the mouse embryos.

PCR reactions were run on a Mastercycler epgradient S (Eppendorf) and product sizes were reveal by agarose gel electrophoresis and ethidium bromide staining.

TAE buffer

80 mM Tris base

1 mM EDTA

0.11% acetic acid

in dwater

***C/EBPβ*$^{-/-}$ mice genotyping primers**

Primer name	Primer sequence
Primer WT-Fw	AGCCCCTACCTGGAGCCGCTCGCG
Primer WT-Rev	GCGCAGGGCGAACGGGAAACCG
Primer KO-Fw	GCTCCAGACTGCCTGGGAAAAG
Primer KO-Rev	GGCCCGGCTAGACAGTTACACG

C/EBPβ$^{-/-}$ mice genotyping PCR

PCR reagents	Stock concentration	End concentration	Amount per probe
dwater			11.65 µl
10x PCR buffer (Invitrogen)	10x	1x	2 µl
DMSO (Roth)	100%	10%	2 µl
dNTP (Fermentas)	10 mM	0.25 mM	0.5 µl
$MgCl_2$ (Invitrogen)	50 mM	2 mM	0.8 µl
Primer WT-Fw	50 µM	1.25 µM	0.5 µl
Primer WT-Rev	50 µM	1.25 µM	0.5 µl
Primer KO-Fw	50 µM	1.25 µM	0.5 µl
Primer KO-Rev	50 µM	1.25 µM	0.5 µl
Taq-polymerase (Invitrogen)	5 U/µl	1.25 units	0.25 µl
DNA template			1 µl

Irf8$^{-/-}$ mice genotyping primers (Holtschke et al., 1996)

Primer name	Primer sequence
A506	CATGGCACTGGTCCAGATGTCTTCC
A540	CTTCCAGGGGATACGGAACATGGTC
A541	CGAAGGAGCAAAGCTGCTATTGGCC

Irf8$^{-/-}$ mice genotyping PCR

PCR reagents	Stock concentration	End concentration	Amount per probe
dwater			11.4 µl
10x PCR buffer	10x	1x	2.5 µl
DMSO	100%	10%	2.5 µl
dNTP	10 mM	0.2 mM	0.5 µl
$MgCl_2$	50 mM	1.25 mM	0.625 µl
Primer A506	50 µM	0.5 µM	0.25 µl
Primer A540	50 µM	0.5 µM	0.25 µl
Primer A541	50 µM	0.5 µM	0.25 µl
Taq-polymerase	5 U/µl	1.25 units	0.25 µl
DNA template			1 µl

PCR program	*C/EBPβ*$^{-/-}$	*Irf8*$^{-/-}$
95°C	90''	4'
95°C	30''	1'
annealing	30'' (55°C)	1' (64°C)
72°C	1'	1'
cycles	40	36
72°C	5'	10'
4°C	HOLD	HOLD
PCR products:		
WT	283 bp	259 bp
KO	351 bp	547 bp

Agarose gel

1.5% agarose (w/v) (Bio&SELL) in TAE buffer

2.1.2 Cell preparation and cell sorting

To obtain primary B cell progenitors, BM cells from 3-6 months old C57BL/6, *C/EBPβ*$^{-/-}$ or *Irf8*$^{-/-}$ mice were flushed from hind leg bones. Cell suspension was subjected to erylyses with ACK buffer and cell aggregates were removed through filtering through 30 µm pre-separation filter (Miltenyi Biotec). Cells were washed twice in DPBS (Invitrogen), resuspended in MACS buffer and incubated with biotinilated antibodies against Gr-1 (RB6-8C5), CD11b (M1/70), CD4 (GK1.5), CD8 (53-6.7), TER-119 (TER-119), and CD49b (DX5) (all from Biolegend) (12.5 µl antibody / 10^8 cells). The code given in parentheses designates the clone from which each antibody is derived. The lineage positive (Lin^+) cells were depleted using Dynabeads sheep anti-Rat IgG (Invitrogen) and magnet stand DynaMag-15 (Invitrogen). The remaining Lin^- cells were then stained with rat anti-mouse B220-PE Cy7 (RA3-6B2), CD19–FITC (6D5), Gr-1-PE (RB6-8C5), SA-APC Cy7 (all purchased from Biolegend), IgM–APC (II/41, BD), and 0.4 µM DAPI (Invitrogen, Molecular probes) to exclude dead cells. Lin^- $B220^+$ IgM^- $CD19^{+/-}$ pre-pro/pro/pre B cells, Lin^- $B220^+$ IgM^+ immature B cells and Lin^+ cells were sorted by fluorescence activated cell sorting (FACS) on BD

FACS Area II (BD Biosciences, BCRT Flow Cytometry Lab) (Fig. 2.1A). The purity of all sorted populations was re-checked and confirmed after the sorting (Fig. 2.1B).

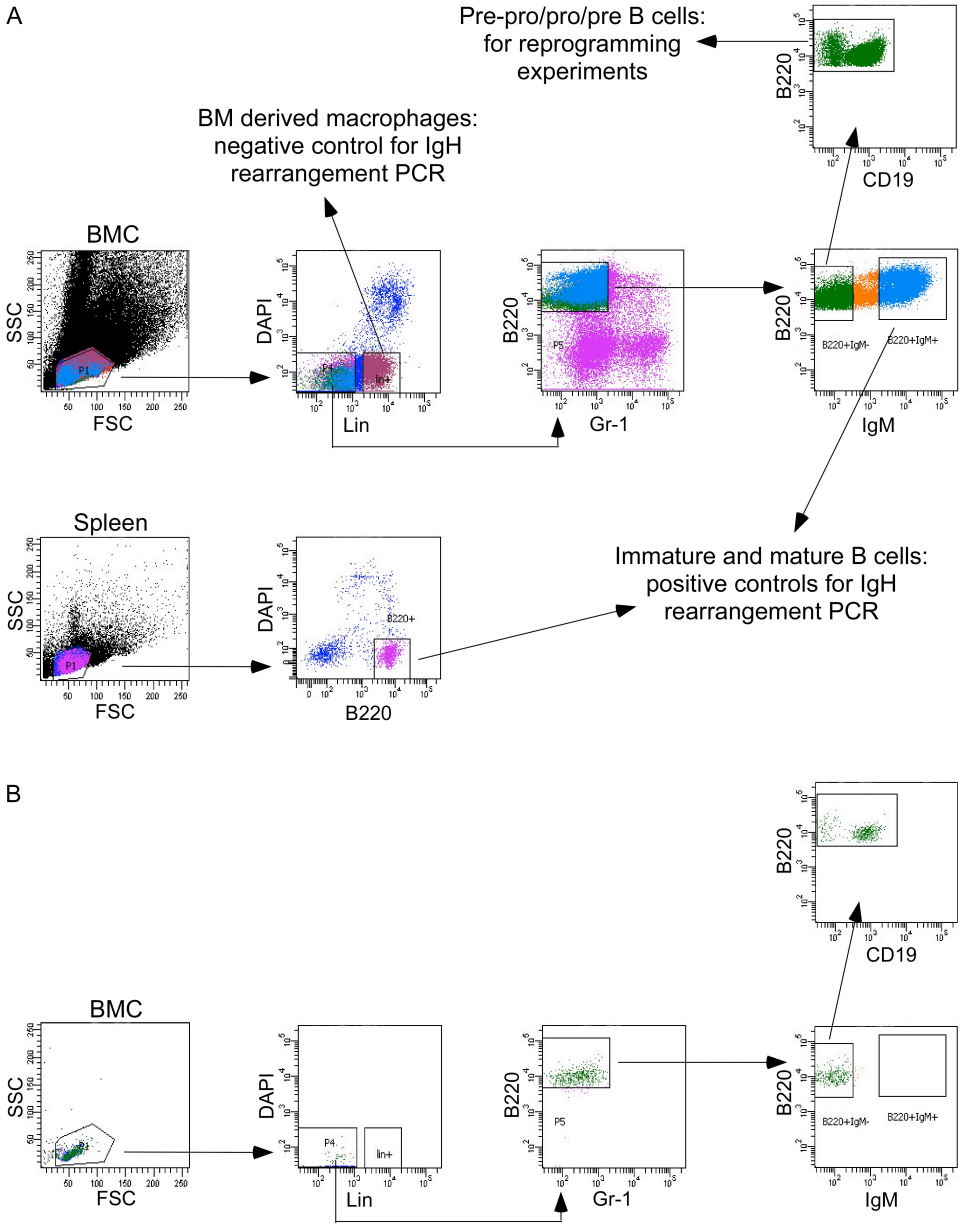


Fig. 2.1 FACS sorting strategy.

A. BM single cell suspension was prepared as described. Cells were stained with lineage cocktail biotinilated antibodies, B220-PE Cy7, CD19-FITC, IgM-APC, Gr-1-PE, SA-APC Cy7 and DAPI. Lin^- $B220^+$ IgM^- $CD19^{+/-}$ pre-pro/pro/pre B cell progenitors were sorted for the reprogramming experiments. Lin^+ cells were cultured for 7-10 days supplemented with M-CSF for obtaining BM derived macrophages for negative controls for IgH rearrangement PCR. Lin^- $B220^+$ IgM^+ immature B cells and spleen $B220^+$ B cells were sorted for positive rearrangement PCR controls.

B. Reanalysis of the sorted pre-pro/pro/pre B cell progenitors population confirmed their purity.

For the spleen B cell preparation, splenic single cell suspensions were prepared using 100 µm cell strainer (BD). After erythrocyte lysis and blocking with rat anti-mouse CD16/32 antibody (Cl. 2.4G2, BD Pharmingen), cells were stained with B220-FITC (RA3-6B2) and 0.4 µM DAPI and $B220^{+}$ $DAPI^{-}$ mature B cells were FACS sorted (Fig. 2.1A).

Erythrocyte lysis buffer (ACK buffer)

Content	Concentration
NH_4Cl	0.15 M
$KHCO_3$	1 M
EDTA	0.1 mM
in dwater	
Adjust pH to 7.2-7.4	

MACS buffer

Content	Concentration
BSA (Sigma)	2%
EDTA	2 mM
in PBS	

2.1.3 Isolation of Fetal Liver (FL) Cells

Mouse embryos were generated from matings between *C/EBPβ*$^{+/-}$ male and female mice. Detection of the vaginal plug was designated as E0.5 dpc (days post coitum). Pregnant females were sacrificed at E14.5 dpc. Embryos were separated from maternal tissue and placed in a Petri dish containing PBS. The FL was removed and a single cell suspension was prepared by passing tissue through a 1 ml pipette tip. *C/EBPβ* genotyping PCR was performed as described in section 2.1.1.

2.2 Retroviral vectors and retrovirus production

2.2.1 Retroviral vectors

The C/EBPβ expression plasmids are based on the chicken sequence (gene bank # EMBLZ 21646). The C/EBPβ LAP* start site was optimized to a Kozak consensus sequence by exchanging a glutamine at position 2 to glutamic acid. Construction of

CR-deletion mutants was published earlier (Kowenz-Leutz et al., 1994). CR1 covers AA 1-13; CR2 to AA 18-38; CR3 AA 42-63; CR4 to AA 99-113; CR 5 to AA 184-222; CR6 to AA 145-179; CR7 to AA 184-222 and CR8-9 (bZIP) to AA 243-317.

C/EBPβ point mutations were done by site directed mutagenesis with the QuikChange site-directed mutagenesis kit (Stratagene). Mutations were confirmed by sequencing. All C/EBPβ expression plasmids were originally generated in the pcDNA3 plasmid backbone (Invitrogen) by 5'-end HindIII /EcoRI and 3'-end XbaI ligation. For introduction of the C/EBPβ constructs into the B cell progenitors, C/EBPβ WT isoforms and C/EBPβ deletion and point mutants were subcloned into MIEG3 (an improved murine stem cell virus (MSCV)-based bi-cistronic retroviral giving a very bright EGFP fluorescence (Williams et al., 2000)) by a 5'-end EcoRI ligation of vector and insert and the 3'-end using a fill-in reaction of the XhoI MIEG3 vector site and 3'-end C/EBPβ insert XbaI site by Klenow followed by a blunt end ligation. Correct insertions of C/EBPβ variants were verified by sequencing and protein expression of the constructs was controlled by western blot. The rat C/EBPα p42 and p30 isoforms where cloned into the MIEG3 vector as described for C/EBPβ.

2.2.2 Bacteria transformation

Chemically competent E. coli bacteria TOP10F' (Invitrogen) were used to generate DNA plasmid. Bacteria were transformed with plasmid DNA using the heat-shock protocol. Competent bacteria were mixed and incubated with 100 ng plasmid DNA on ice for 30 min. Mixture was incubated for 90 sec at 42°C and quickly chilled on ice for 2 minute. Then 1 ml LB medium without antibiotics was added and the suspension was incubated for 1 hour at 37°C under gentle shaking. After that bacteria were pelleted (3 min, 6000 rpm), supernatant was removed and bacteria were plated onto LB agar plates containing 200 µg/ml ampicillin (Roth). Plates were incubated overnight at 37°C. Colonies were picked and grown in LB medium with antibiotics under agitation (200 rpm, overnight, at 37°C). Transformed bacteria were maintained as glycerol stocks in 15% glycerol and stored at -80°C.

2.2.3 Plasmid DNA isolation

Plasmid DNA was extracted using NucleoBond Xtra Midi (Macherey-Nagel). LB medium was inoculated with either one colony from the LB agar plate or with bacterial pre-culture. The culture was incubated at 37° C overnight at 200 rpm on a

shaker. After peletting, bacteria were resuspended in 8 ml resuspension buffer RES containing RNase. Then 8ml lysis buffer LYS was added to the suspension and incubated for 5 min at room temperature (RT). Subsequently, lysis was stopped with 8 ml neutralization buffer NEU and lysate was immediately loaded on equilibrated NucleoBond Xtra Column Filter. After loading, the filter and the column were washed twice and the filter was discarded. Plasmid DNA was eluted with 5 ml elution buffer ELU. The eluate was precipitated with isopropanol and centrifuged (30 min, 4°C, 15000 x g). The plasmid DNA pellet was washed with 70% ethanol, airdried and finally resuspended in sterile dwater.

2.2.4 Retrovirus production

Viruses were produced by transfection of Plat-E cells (Morita et al., 2000), which before transfection were under constant selection with 1 µg/ml Puromycin (PAA) and 10 µg/ml Blasticidin (PAA) for removing cells which had lost the gagpol and env transgenes. In the morning of the day of the transfection 3 x 10^6 cells Plat-E cells were plated in 10 cm dish in medium DMEM + Glutamax (Invitrogen), 10% hiFCS (FCS (Invitrogen) heat inactivated for 1h, 56°C), Penicillin-Streptomycin (PAA) and incubated at 37°C and 5% CO_2 (Binder incubator). In the late afternoon the transfection mix was prepared in 1.5 ml tube as follows:

Component	Amount per transfection
retroviral vector pMSCV	5 µg
pGagpol	10 µg
pEnv	2 µg
H_2O	up to 450 µl
2.5M $CaCl_2$	50 µl

This DNA/$CaCl_2$ mix was added to 500 µl 2xHEBS buffer in 15 ml tube drop wise while vortexing under the hood and incubated 20 min at RT. Meanwhile, the medium from the 10 cm dishes with the plated Plat-E cells was replaced with 9 ml fresh complete DMEM to which 25 µM chloroquine (Sigma-Aldrich) was added. The DNA/$CaCl_2$/HEBS mix was carefully added to the cells while the plate was gently being swirled. The transfected cells were incubated overnight at 37°C and 5% CO_2.

On the next morning the medium was removed and 8 ml complete DMEM without chloroquine was added. Plates were incubated at 32°C and 5% CO_2 for the virus production and the virus supernatants were harvested every 48h after the transfection for one week, filtered through 0.45 µm filter and stored at -80°C.

2xHEBS buffer

Content	Concentration
HEPES	50 mM
NaCl	280 mM
Na_2HPO_4	1.5 mM
in ddwater	
Adjust pH to 7.05 +/-0.05	

The transfection reagent stock solutions 2xHEBS buffer, 2.5 M $CaCl_2$ and 25 mM chloroquine were sterile filtered through 0.22 µm filter and stored at -20°C.
To test the quality of the produced virus, aliquots of the supernatants were used to infect 70Z/3 murine pre B cell line (gift from C. Scheidereit). 1 x 10^5 70Z/3 cells were plated in 500 µl medium RPMI 1640 (Invitrogen), 10% hiFCS (Invitrogen), Penicillin-Streptomycin (PAA), 50 µM 2-mercaptoethanol (Invitrogen) and infected with equal volume retroviral supernatant to which 8 µg/ml polybrene (Sigma) was added. Cells were incubated for 3 - 5h at 37°C and 5% CO_2, washed and analyzed 48h post-infection by FACS for the percentage of GFP^+ PI^- / $7\text{-}AAD^-$ (PI (Sigma) end concentration 1 µg/ml, 7-AAD (BD Pharmingen) end concentration 0.5 µg/ml) on Accuri C6 Cytometer, CFlow plus Analysis software (Accuri). Only supernatants that led to infection of more that 10% of 70Z/3 cells were used for the infection of the primary B cells.

2.3 Cell infection, cultivation and CFU assay

2.3.1 B cell progenitors infection and cell cultivation

For the virus infection, purified B cell progenitors were resuspended at 1.5 - 2 x 10^5 cells/ml in medium IMDM + Glutamax with 20% hiFCS, Penicillin-Streptomycin (PAA), 1 mM Sodium Pyruvate (PAA), 50 µM 2-mercaptoethanol (Invitrogen) as well as 10 ng/ml IL-7, SCF, Flt-3L (all purchased from Peprotech). Cells were deposited

into 24-well plates and infected twice with equal volume retroviral supernatant to which 8 µg/ml polybrene (Sigma) was added. Plates were centrifuges for 60-90 min at 2100 rpm at 32°C in an Eppendorf centrifuge 5810R followed by 3-5h incubation at 32°C. Infected cells were transferred into polystyrene 0.4 µm pore membrane HTS Transwell-24 well (Corning) supplemented with 10 ng/ml IL-7, SCF, Flt-3L, IL-3 and M-CSF (Peprotech) (Xie et al., 2004) and co-cultured with S17 (gift from T. Graf) stromal cells (10 µg/ml mitomycin (Sigma) treated). Fresh medium was added every other day.

2.3.2 BM derived macrophages

For obtaining BM derived macrophages, Lin^+ cells were sorted during the sorting of B cell progenitors (Fig. 2.1A). Cells were cultured in IMDM medium, 20% hiFCS supplemented with 10 ng/ml recombinant murine M-CSF (Peprotech) for 6-9 days, medium was changes every 2-3 days.

2.3.3 CFU assays

2 x 10^4 FL cells/well/1ml were plated in triplicates in methylcellulose MethoCult GF M3434 (Stem Cell Technologies), containing 50 ng/ml SCF, 10 ng/ml IL-3, 10 ng/ml IL-6, 3 U/mL EPO, or MethoCult M3234 (Stem Cell Technologies), containing a distinct cytokine: 10 ng/ml M-CSF, or 50 ng/ml G-CSF, or 20 ng/ml GM-CSF. Plates were incubated for 1 week at 37°C and total colony and differentiation counts for granulocyte-erythrocyte-macrophage-megakaryocyte (CFU-GEMM), granulocyte-macrophage colonies (CFU-GM), macrophage colonies (CFU-M), granulocyte colonies (CFU-G) and burst forming unit erythroid colony formation (BFU-E) were performed.

2.4 FACS analyses

2.4.1 FACS analyses for characterization of lymphoid to myeloid lineage reprogramming

Before the FACS analyses, 6 or 9 days *in vitro* reprogrammed cells were detached from the tissue culture plates using accutase (PAA), washed and resuspended in FACS buffer. After Fc blocking with rat anti-mouse CD16/32 antibody (Clone 2.4G2, BD Pharmingen) cells were stained with rat anti-mouse CD11b-PerCP Cy5.5

(M1/70), CD11b-APC Cy7 (M1/70), CD11b-PE (M1/70), CD19-APC (6D5), CD19-biotin (6D5), CD19-PE Cy7 (6D5), CD45-PE Cy7 (30-F11), Gr-1-APC Cy7 (RB6-8C5), Ly-6C-APC Cy7 (HK1.4), Ly-6G-APC (1A8), CD115-PE (M-CSFR, Cl. AFS98), CD115-APC (M-CSFR, Cl. AFS98), F4/80-Pacific blue (Cl:A3-1), MHC-II (I-A/I-E-PE, Cl. M5/114.15.2), SA-PE (all from Biolegend), CD86-PE (B7-2), hamster anti-mouse CD11c-APC (HL3) and CD11c-V450 (HL3) (BD Pharmingen) and 7-AAD (BD Pharmingen) or DAPI (Invitrogen, Molecular probes) were added to discriminate cell viability. Samples were run on FACS Canto II machine (BD Biosciences, BD Diva Software) and analyzed with FlowJo software.

FACS buffer

Content	Concentration
hiFCS	2%
EDTA	2 mM
NaN_3	0.1%
in PBS	

2.4.2 Intracellular protein staining

For the intracellular C/EBPβ protein staining, 6 days after the infection cells were incubated with Mouse BD Fc Block (Clone 2.4G2, BD Pharmingen) followed by CD11b-APC Cy7 (M1/70), CD19-APC (6D5), CD45-PE Cy7 (30-F11) (Biolegend) surface marker staining. Cells were washed and then fixed and permeabilized with BD Cytofix/Cytoperm Buffer for 20 min at 4°C. After blocking with 8% donkey serum (Jackson Immuno Research) for 30min at 4°C, cells were stained with rabbit anti C/EBPβ antibody (Santa Cruz, C-19) at 4°C for 1h at dark and then with anti - rabbit PE conjugated antibody (Jackson Immuno Research) at 4°C for 30 min at dark. Cells were washed and resuspended in FACS staining buffer prior to FACS analyses. All antibody dilutions, serum blocking and washing steps were performed in 1x Perm Wash Buffer (BD). The relative C/EBPβ expression in the virus infected cells was calculated as a delta (Mean) of C/EBPβ fluorescence of the probes incubated with or without the primary C/EBPβ antibody and after subtraction of the endogenous expression for each sample.

2.4.3 AnnexinV staining

For AnnexinV staining, 6 days after the infection, cells were incubated with Mouse BD Fc Block (BD Pharmingen) and stained for surface marker expression. Cells were washed twice with PBS, up to 10^5 cells were resuspended in 100 µl 1x AnnexinV binding buffer (Biolegend) and stained with AnnexinV-APC (BD Pharmingen). After 15 min incubation at RT at dark, 300 µl 1x AnnexinV binding buffer and DAPI at final concentration 0.4 µM were added to the stained cells prior to FACS analyses. Cells were defined as follows: live cells (AnnexinV$^-$ DAPI$^-$), early apoptotic (AnnexinV$^+$ DAPI$^-$) and late apoptotic and/or necrotic cells (AnnexinV$^+$ DAPI$^+$).

2.4.4 Phagocytosis assay

For the phagocytosis assay, 10 days after the infection, cells were washed twice with PBS and equilibrated for 1h at 37°C in Opti-MEM (Invitrogen) without FCS in 24 well tissue culture plates supplemented with 10 ng/ml M-CSF. Cells were cooled to 4°C and pre-incubated with 1 µl 1.0 µm Blue (365/415) fluorescent carboxylate-modified microspheres (Molecular Probes). After washing cells were incubated for 1h at 37°C, detached with accutase (PAA) and incubated with Mouse BD Fc Block (BD Pharmingen) for 20 min at 4°C. Cells were then stained with CD45-PE Cy7 (30-F11), CD11b-PerCP Cy5.5 (M1/70), CD19-APC (6D5) (Biolegend), fixed with 1% paraformaldehyde in PBS for 20 min at 4°C, washed with FACS buffer and FACS analyzed.

2.5 Cytospins and Immunoglobulin Gene Rearrangements

2.5.1 Cytospins and May-Grunwald / Giemsa staining

Cell morphology was assessed by spinning 10 days reprogrammed FACS sorted GFP$^+$ CD11b$^+$ and GFP$^+$ CD19$^+$ cells in a cytospin-centrifuge (Cytospin4, Thermo biosciences) at medium acceleration, 800 rpm for 5 min on a glass slide, followed by an air-drying step. Cells were fixed in Methanol for 5 min and subsequently subjected to May-Grunwald staining (Sigma-Aldrich) for 5 min. After a brief washing step in dwater, a second staining with Giemsa stain (Sigma-Aldrich) for 40 min was performed. Slides were washed thoroughly with dwater, dried, mounted with Entellan (Merck) and covered with a cover glass. Cells were photographed with an Axio

Scope.A1 microscope/camera system (Zeiss), AxioVision Rel. 4.7 software, 63x objective with immersion oil 518 N (Zeiss).

2.5.2 Immunoglobulin Gene Rearrangement PCR

GFP^+ $CD11b^+$ and GFP^+ $CD19^+$ cells from the S17 co-cultures were sorted by FACS 6 or 9 days after the retroviral infection. Genomic DNA was extracted using SDS/proteinase K method as described before for the tailtips. For D_HJ_H rearrangements in IgH gene locus the primer pair D_{FS} and J_H4A was used together with D_Q52 for the germline locus configuration (Ehlich et al., 1994). BM derived macrophages *in vitro* cultured in M-CSF supplemented medium (negative control) and splenic B cells (positive control) were used as controls.

IgH Gene Rearrangement PCR primers (Ehlich et al., 1994)

Primer name	Primer sequence
5' D_{FS} (Fw1)	ACGTCGACTTTTGT(GC)AAGGGATCTACTACTGT
5' D_Q52 (Fw2)	ACGTCGACGCGGACGACCACAGTGCAACTG
3' J_H4A (Rev)	GGGTCTAGACTCTCAGCCGGCTCCCTCAGGG

IgH Gene Rearrangement PCR

PCR reagents	Stock concentration	End concentration	Amount per probe
Water			14.4 µl
10x PCR buffer	10x	1x	2 µl
dNTP	10 mM	0.2 mM	0.4 µl
$MgCl_2$	50 mM	1.5 mM	0.6 µl
Primer Fw1	50 µM	1 µM	0.4 µl
Primer Fw2	50 µM	1 µM	0.4 µl
Primer Rev	50 µM	1 µM	0.4 µl
Taq-polymerase	5 U/µl	2 units	0.4 µl
DNA template			1 µl

PCR program	
95°C	5’
95°C	45’’
63°C	45’’
72°C	90’’
cycles	35
72°C	8’
4°C	HOLD
PCR products:	
Germline configuration	2150 bp
D_HJ_H1	1460 bp
D_HJ_H2	1150 bp
D_HJ_H3	730 bp
D_HJ_H4	200 bp

Agarose gel

1.25% agarose (w/v) in TAE buffer

2.6 Protein and RNA analyses

2.6.1 Cell lysis and western blotting

For the protein analyses, cells were lysed in RIPA buffer “upstate” supplemented with protease inhibitors and incubated for 1h on ice and every 10 min subjected to sonication (Bandelin). Cells were then centrifuged at 4°C for 30 min at 12.000 rpm in a microcentrifuge 5417R (Eppendorf) and the supernatant aspirated and placed in a fresh tube on ice, while pellet was discarded. Total protein concentration of individual samples was estimated using Bradford reagent (Sigma) and obtained from photometric analysis at 595 nm wavelength on NanoDrop™ 1000 Spectrophotometer (Thermo Scientific) and calculated based on a standard Bovine serum albumin (BSA) curve. After that extracts were boiled for 5 min in 1x SDS-loading buffer, incubated for 5 min on ice and stored at -80°C or immediately used for electrophoresis.

Proteins were separated on 10% SDS-polyacrylamide gels (run at 120 V until the bromophenol blue dye reached the end of the gel) in mini vertical gel electrophoresis

unit (Amersham Biosciences). In parallel to the probes, BenchMark Pre-Stained Protein Ladder (Invitrogen) was loaded and used for protein molecular weight estimation. Proteins were then electrotransferred onto nitrocellulose membranes (Millipore) in wet-blotting tank electrophoresis unit (Amersham Biosciences) for 1h at 400 mA in Transfer buffer at constant stirring. Unspecific binding of immunoglobulin was reduced by incubating membranes in 1x Roti-Block (Roth) for 1h 30min at RT. Subsequently membranes were probed with polyclonal rabbit anti- C/EBPβ (Santa Cruz, C-19 or Leutz lab) or polyclonal rabbit anti- C/EBPα (Santa Cruz, 14AA) and monoclonal mouse anti- α-tubulin antibody (Santa Cruz, TU-02) diluted in 1x Roti-Block overnight at 4°C at constant shaking. After thoroughly washing with 1xTBST, blots were incubated for 2h at RT with IRDye™ 680 Goat anti-rabbit IgG and IRDye™ 800CW Goat anti-mouse IgG (LI-COR) diluted in 1x Roti-Block and scanned with Odyssey Infrared Imaging System (LI-COR).

Ripa buffer "upstate"

Content	Concentration
Tris-HCL, pH 7.4	50 mM
NaCl	150 mM
NP-40	1%
Sodium Deoxycholat	0.25%
SDS	0.1%
EDTA	1 mM
in dd water	
Proteinase inhibitors	
Na_3VO_4	1 mM
phenanthroline	1 mM
DTT	1 mM
PMSF	1 mM
pepstatin (AppliChem)	10 µg/ml
aprotenin A (AppliChem)	10 µg/ml
leupeptin (AppliChem)	10 µg/ml

SDS loading buffer (6x)

Content	Concentration
DTT	600 mM
Tris pH6.8	350 mM
SDS	10%
glycerol	10%
bromphenol blue	0,1 mg/ml
in dd water	

Polyacrylamid gels

Content	10% separating gel (ml)	stacking gel (ml)
Acrylamid (30%)/Bisacrylamid (0.8%) (Roth)	5 ml	0.65 ml
4x Tris-HCl/ 0.4% SDS	3.75 ml (pH 8.8)	1.25 ml (pH 6.8)
H_2O	6.25 ml	3.05 ml
10% APS (Bio-Rad)	0.060 ml	0.036 ml
TEMED (Roth)	0.012 ml	0.006 ml

Running buffer

Content	Concentration
Tris base	25 mM
glycine	200 mM
SDS	0.1%
in ddwater	

Transfer buffer

Content	Concentration
Tris base	25 mM
glycine	200 mM
SDS	0.1%
methanol	20%
in ddwater	

TBST buffer

Content	Concentration
Tris-HCl, pH 7.4	50 mM
NaCl	150 mM
Tween-20	0.1%
in ddwater	

2.6.2 RNA extraction and mRNA expression analyses by Nanostring technology

Total RNA was extracted 6 days after infection of *C/EBPβ*$^{-/-}$ B cell progenitors with C/EBPβ constructs and from BM derived macrophages (after 6 days *in vitro* culturing) using RNeasy Micro Kit (QIAGEN) according to the manufacture's recommendations. In brief, cells were lysed in RLT buffer, containing 150 mM 2-mercaptoethanol. After adding an equal volume of 70% ethanol, the sample was loaded on RNeasy MinElute spin column and briefly centrifuged. The flow through was discarded and the column was washed with Buffer RW1. DNA was digested with RNase free DNase set (QIAGEN) for 15 min at RT. After washing of the column with buffer RW1, buffer RPE and 80% ethanol, total RNA was eluted in RNase-free water. RNA concentration and purity was measured on NanoDrop^{TM} 1000 Spectrophotometer (Thermo Scientific) at 260 nm (A260) and, additionally, RNA integrity was measured on Agilent 2100 Bioanalyzer (Agilent Technologies). Only samples with A260/A280 = 1.9 - 2.1, RNA integrity number (RIN) >7 and RNA concentration >15 ng/µl were used for subsequent applications.

Nanostring technology is described elsewhere (Fortina and Surrey, 2008). Briefly, the method involves mixing total RNA with pairs of capture and reporter probes custom-synthesized for each mRNA of interest. After hybridization, excess reporters and capture probes were removed and probe-bound mRNAs was immobilized and color-coded bar tags on the reporter probes were scanned and decoded. Synthesis of the oligonucleotide probes and Nanostring RNA expression analyses were performed by nCounter, Heidelberg. Table 2.1 represents the list of the targeted sequences in the analyzed genes. To determine mRNA expression levels, background was subtracted, all negative values were set to 1 for each gene of interest and the positive values were normalized to three house-keeping genes (*Gapdh*, *Tbp* and *Ppia*).

2.7 Statistical analyses

In all experiments, data are presented as mean ± SEM (standard error of the mean). Statistical analyses were done on Prism 4.0a (GraphPad Software) using unpaired two-tailed t test for the calculation of the P-value. The statistical significance of the P-value was defines as: $P>0.05$ - not significant, $P=0.01$-0.05 - significant (*), $P=0.001$-0.01 - very significant (**), $P<0.001$ - extremely significant (***).

Table 2.1

Targeted sequences in the selected genes whose expression was analyzed in the reprogrammed cells and BM derived macrophages by NanoString technology; HKG - house keeping genes.

	Gene symbol	Target ID	Targeted Region	Target Sequence 5'-3'
M1 genes	*Tnf* (*Tnfa*)	NM_013693.1	1135-1235	TTCCTGAGTTCTGCAAAGGGAGAGTGGTCAGGTTGCCTCTGTCTCAGAATGAGGCTGGATAAGATCTCAGGCCTTCCTACCTTCAGACCTTTCCAGACTC
	Il1b	NM_008361.3	1120-1220	GTTGATTCAAGGGGACATTAGGCAGCACTCTCTAGAACAGAACCTAGCTGTCAACGTGTGGGGGATGAATTGGTCATAGCCCGCACTGAGGTCTTTCATT
	Il6	NM_031168.1	40-140	CTCTCTGCAAGAGACTTCCATCCAGTTGCCTTCTTGGGACTGATGCTGGTGACAACCACGGCCTTCCCTACTTCACAAGTCCGGAGAGGAGACTTCACAG
	Il12b	NM_008352.1	1045-1145	TCGTAGAGAAGACATCTACCGAAGTCCAATGCAAAGGCGGGAATGTCTGCGTGCAAGCTCAGGATCGCTATTACAATTCCTCGTGCAGCAAGTGGGCATG
	Il12rb1	NM_008353.2	2765-2865	AAAGCAGGGCCTAGACATTCACGGGAAGTTTATACGTCTGGACTCAGTTTCCCTATTAGAGTATTGGGCACTTAATAAATGGGCCTTCCCAGAGACTGAG
	Ccr7	NM_007719.2	755-855	CCCAGATGGTTTTTGGGTTCCTAGTGCCTATGCTGGCTATGAGTTTCTGCTACCTCATTATCATCCGTACCTTGCTCCAGGCACGCAACTTTGAGCGGAA
	Cxcl9	NM_008599.2	40-140	TAGAACTCAGCTCTGCCATGAAGTCCGCTGTTCTTTTCCTCTTGGGCATCATCTTCCTGGAGCAGTGTGGAGTTCGAGGAACCCTAGTGATAAGGAATGC
	Cxcl10	NM_021274.1	115-215	AGGACGGTCCGCTGCAACTGCATCCATATCGATGACGGGCCAGTGAGAATGAGGGCCATAGGGAAGCTTGAAATCATCCCTGCGAGCCTATCCTGCCCAC
	Ccl2 (*Mcp-1*)	NM_011333.3	415-515	TCTTCAGCACCTTTGAATGTGAAGTTGACCCGTAAATCTGAAGCTAATGCATCCACTACCTTTTCCACAACCACCTCAAGCACTTCTGTAGGAGTGACCA
	Mmp8	NM_008611.4	2285-2385	GGAGGGCTGTATCTATAAATCTATTTGCCAATAAGTTCCCAGGCAGAGGCAGGTAGGAGGGGTTTAAAAAAAAGGACCATTCCTTTCTCAAGCACATTCC
	Mmp9	NM_013599.2	1570-1670	CCTCTACAGAGTCTTTGAGTCCGGCAGACAATCCTTGCAATGTGGATGTTTTTGATGCTATTGCTGAGATCCAGGGCGCTCTGCATTTCTTCAAGGACGG
	Nos2	NM_010927.3	3715-3815	CCCCCCTCCTCCACCCTACCAAGTAGTATTGTACTATTGTGGACTACTAAATCTCTCTCCTCTCCTCCCTCCCCTCTCTCCCTTTCCTCCCTTCTTCTCC

M2 genes	*Il10*	NM_010548.1	985-1085	GGGCCCTTTGCTATGGTGTCCTTTCAATTGC TCTCATCCCTGAGTTCAGAGCTCCTAAGAGA GTTGTGAAGAAACTCATGGGTCTTGGGAAG AGAAACCA
	Arg1	NM_007482.3	626-726	GTACATTGGCTTGCGAGACGTAGACCCTGG GGAACACTATATAATAAAAACTCTGGGAATT AAGTATTTCTCCATGACTGAAGTAGACAAGC TGGGGATT
	Il13ra1	NM_133990.4	845-945	CTCAAACCGACCGACATAATATTTTAGAGGT TGAAGAGGACAAATGCCAGAATTCCGAATCT GATAGAAACATGGAGGGTACAAGTTGTTTCC AACTCCC
	Msr1	NM_031195.2	555-655	GATTTCGTCAGTCCAGGAACATGGGAATTCA CTGGATGCAATCTCCAAGTCCTTGCAGAGTC TGAATATGACACTGCTTGATGTTCAACTCCA TACAGAA
	Tgfb1	NM_011577.1	1470-1570	GGAGTTGTACGGCAGTGGCTGAACCAAGGA GACGGAATACAGGGCTTTCGATTCAGCGCT CACTGCTCTTGTGACAGCAAAGATAACAAAC TCCACGTGG
	Il4ra	NM_001008700.3	670-770	TGGAATAACCTGTACCCATCGAACAACTTAC TGTACAAAGACCTCATCTCCATGGTCAACAT CTCCAGAGAGGACAACCCTGCAGAATTCATA GTCTATA
	Alox15	NM_009660.3	815-915	CAGCTGGATGAGGAGCTCAAGAAAGGCACT CTGTTTGAAGCGGATTTCTTCCTTCTGGATG GGATCAAGGCCAATGTCATCCTTTGTAGTCA GCAGTACC
	Mmp12	NM_008605.3	2725-2825	AGGAAAGGGCTCCTTTGCTCCATGTGTCAG ATGTGAGTATTAACCTTCGACATCAACTTCAT GAGATCCAGAGTCATGTAAGAGACATGTGA GCACTACT
	Pparg	NM_011146.1	1060-1160	ACCAAGTGACTCTGCTCAAGTATGGTGTCCA TGAGATCATCTACACGATGCTGGCCTCCCTG ATGAATAAAGATGGAGTCCTCATCTCAGAGG GCCAAGG
	Ccl22	NM_009137.2	1096-1196	CCAAGAATCAACTTCCACCCCTCTTCAACCA CATGCTAGGGTCTTTTACTTTCTCTGCCCCA CACCTTTGACTCCTTGCCTGTGTAGCTGATA GTCGAAG
	Kdm6b (*Jmjd3*)	NM_001017426.1	4915-5015	GGTGAAGAACGTCAAGTCCATTGTGCCCAT GATTCATGTGTCCTGGAACGTCGCTCGAAC GGTCAAGATCAGCGATCCTGACTTGTTCAAG ATGATCAAG
	Chi3l3 (*Ym1*)	NM_009892.1	1196-1296	AGAGGAGCTTTACACAATGATTTGTCCTTGA AACTCTCAGAATAAGATCAAGTTCAACGGTT TTTCCACAGCGCATTCTGCATCATGCTTCCA TGGAGAA
	Fcgr3	NM_010188.5	1175-1275	TCTGACCTCCACCATCCACCATGGCAGGTG CACACAATAAATTAAAATGTCATGTATATTTT TAAACAAGAGACAGGGGCAGGCTAAGGGTT GATGGCAT

Others	*Mafb*	NM_010658.2	2658-2758	TTGAGCCAAACAGCCATTCTTAGAATGTACT AGAAACCCACACATTGGCAACTAACGCTGCA ACTCTCAAGTGTGTCCTTTAGACCAGTGCAT TATATGT
	Maf (*c-maf*)	NM_001025577.2	43-143	CTGGCAATGAACAATTCCGACCTGCCCACC AGTCCCCTGGCCATGGAATATGTTAATGACT TCGATCTGATGAAGTTTGAAGTGAAAAAGGA ACCGGTGG
	Myd88	NM_010851.2	1595-1695	GCTGCAGGCTCAGCTGTTTTCTCCCCAGCA GCGAGGTTTGCATCTTCTTATTCCTTTCACG TTCTCTACCATAGAGGCAATGTCATGGTCCC TCTCAGGG
	Kdm4a (*Jmjd2a*)	NM_172382.2	1675-1775	CTGTAGGAGGACGCCTCGTCTTCTCGGGTT CCAAAAAGAAATCATCTTCCAGCCTGGGCTC CACTTCATCTCAGGATTCAGTTTCTTCAGATT CTGAAAC
	Ddr2	NM_022563.2	595-695	TGCTTGATGGAAACAGTAACCCTTATGATGT ATTCCTGAAGGACTTGGAGCCACCCATCGT CGCCAGATTTGTTCGCCTTATCCCAGTCACT GACCACTC
HKG	*Gapdh*	NM_008084.1	755-855	ATGTGTCCGTCGTGGATCTGACGTGCCGCC TGGAGAAACCTGCCAAGTATGATGACATCAA GAAGGTGGTGAAGCAGGCATCTGAGGGCCC ACTGAAGGG
	Tbp	NM_013684.3	70-170	GTGGCGGGTATCTGCTGGCGGTTTGGCTAG GTTTCTGCGGTCGCGTCATTTTCTCCGCAGT GCCCAGCATCACTATTTCATGGTGTGTGAAG ATAACCCA
	Ppia	NM_008907.1	390-490	CCAAGACTGAATGGCTGGATGGCAAGCATG TGGTCTTTGGGAAGGTGAAAGAAGGCATGA ACATTGTGGAAGCCATGGAGCGTTTTGGGT CCAGGAATGG

3. Results

3.1 B cell to myeloid reprogramming potential resides in the TAD of C/EBPβ

Seven short peptide CRs in the TAD (CR1-4) and the RD (CR5-7), which are interrupted by LCRs, have been identified in the N-terminus of the transcription factor C/EBPβ (Kowenz-Leutz et al., 1994). An extensive set of approximately thirty different C/EBPβ constructs, including the three WT isoforms, as well as C/EBPβ CR deletion and PTM site mutants (Fig. 3.1, left panel, 3.3, left panel and data not shown) was used to identify the structures in C/EBPβ required for lympho-myeloid trans-differentiation.

A retroviral approach was used to express the set of WT isoforms of C/EBPβ and its mutants in primary B cell progenitors. An early B cell progenitor compartment from murine BM, containing pre-pro, pro and pre B cells, was isolated by FACS sorting as Lin^{-} $B220^{+}$ IgM^{-} $CD19^{+/-}$ cell population (Fig. 2.1). Infected cells were co-cultured with S17 BM stromal cells under conditions that support both B cell and myeloid cell development (Xie et al., 2004) and analyzed by FACS at 6 and 9 days post-infection. The percentage of infected GFP^{+} cells was in most of the experiments between 20 and 50% (data not shown) and all analyses were performed only on these infected cells. No significant change in the B cell phenotype was observed in cells infected with the LIP C/EBPβ isoform, similarly to cells infected with MSCV vector or uninfected controls (Fig. 3.1 and 3.2). In contrast, both the LAP and LAP* C/EBPβ isoforms upregulated the myeloid surface marker CD11b and downregulated the B cell marker CD19, giving rise to double positive $CD19^{+}$ $CD11b^{+}$, as well as $CD19^{-}$ $CD11b^{+}$ cells on day 6 post-infection and almost exclusively $CD19^{-}$ $CD11b^{+}$ cells 9 days after infection, indicating completion of the myeloid trans-differentiation process and loss of the B cell phenotype.

The importance of each CR as an essential unit in C/EBPβ reprogramming was examined by deletion analysis (Fig. 3.1). Comparison between the FACS analyses for CD11b myeloid marker upregulation and CD19 lymphoid marker downregulation by the different C/EBPβ deletion constructs revealed that the RD (CR5,6,7) or an exchange of the leucine zipper dimerization domain, as in the mutant CREB LZ, are dispensable for reprogramming and these mutants had reprogramming properties

undistinguishable from WT C/EBPβ LAP*. Removal of CR5 or CR7 or the entire RD (ΔCR5,6,7) was previously found to enhance the transactivation function of C/EBPβ in various cell types (Kowenz-Leutz et al., 1994; Williams et al., 1995). Moreover, ΔCR6, which represents a dominant negative mutant in fibroblasts (Kowenz-Leutz et al., 1994), displayed trans-differentiation activity similar to the extended WT LAP* C/EBPβ isoform. This suggests that regulation of C/EBPβ activity in B cells differs from regulation in fibroblasts and in erythroid cells (Kowenz-Leutz et al., 1994). Furthermore, the mutant with replaced leucine zipper dimerization domain (CREB LZ) was indistinguishable from WT LAP*, suggesting that the bZip structure plays a minor role during trans-differentiation.

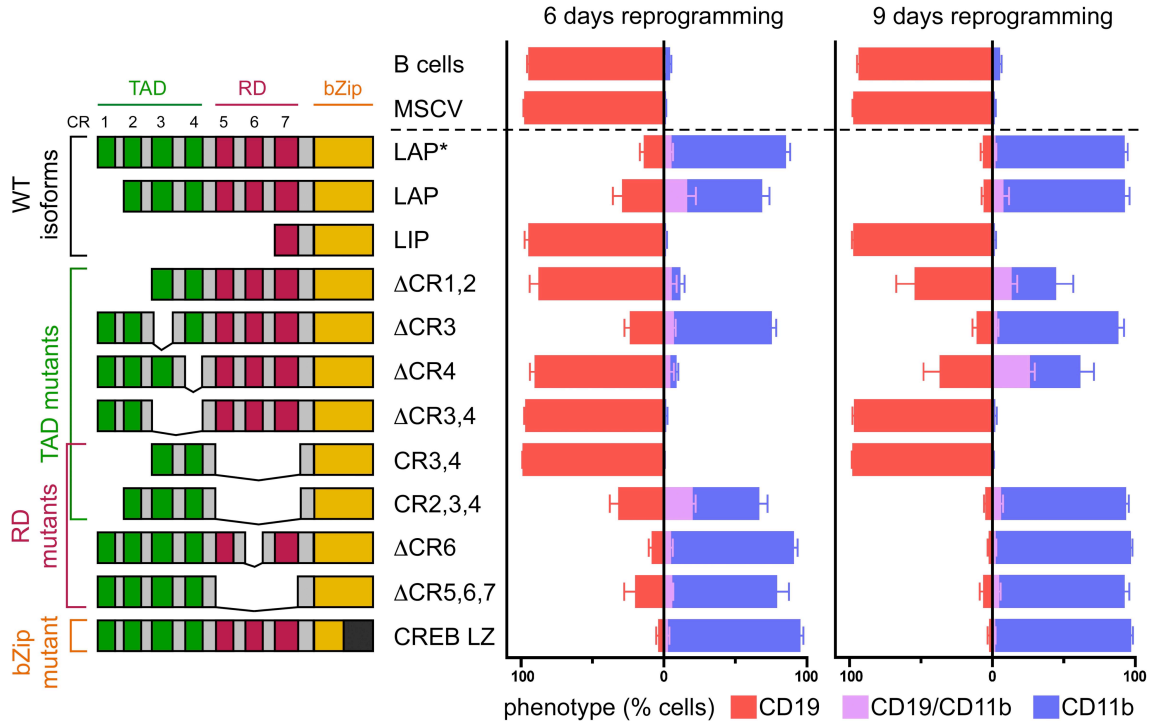


Fig. 3.1 Structural requirements for B cell to myeloid reprogramming potential of C/EBPβ.
Schematic representation of the different C/EBPβ constructs (left) indicating the conserved regions (CRs) in the transactivation domain (TAD; CR1,2,3,4; green), regulatory domain (RD; CR5,6,7; red), bZip domain (yellow), and the low complexity regions (LCRs, grey). Expression of lineage specific markers: B cell CD19 (red), myeloid CD11b (blue), or double positive (magenta) at 6 (middle panel) or 9 days (right panel) after the infection. Bar graph shows percentage of GFP^+ gated (virus infected) cell population; B cells - control uninfected GFP^- B cell progenitors. Results represent mean ± SEM from at least two experiments.

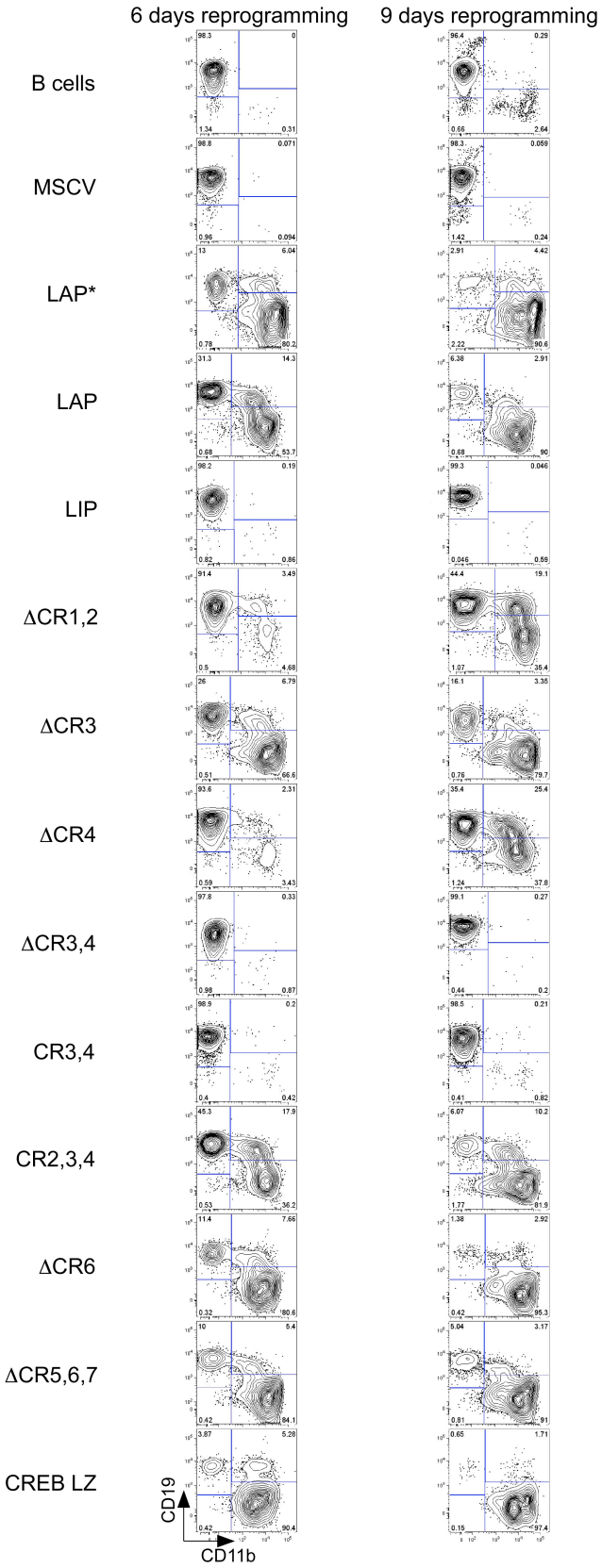


Fig. 3.2 Reprogramming of B cell progenitors by C/EBPβ WT isoforms and deletion mutants. Representative FACS profiles of the infected B cell progenitors at 6 and 9 days after the infection. FACS plots represent GFP^+ gated cell population, B cells - control uninfected GFP^- B cell progenitors. Similar outcomes were obtained from at least two repeat experiments.

The data showed that C/EBPβ reprogramming function resides in its N-terminal TAD. While distinct deletions in the TAD kept, though decreased, reprogramming functions, certain others completely abrogated reprogramming (Fig. 3.1). For example, the deletion mutant ΔCR3 showed comparable to C/EBPβ LAP* reprogramming capacity. Other mutants, like Δ CR1,2 and Δ CR4, demonstrated strongly compromised reprogramming function and compared to LAP* induced a significantly lower percentage of $CD11b^+$ cells after both 6 and 9 days reprogramming (ΔCR1,2 (N=5): day 6 - P<0.0001, day 9 - P<0.0001; ΔCR4 (N=4): day 6 - P<0.0001, day 9 - P=0.0002). On the other hand, the percentage of $CD11b^+$ cells induced by LAP and CR2,3,4 (both proteins lack CR1) was significantly lower than LAP* (N=14) only after 6 days reprogramming but not after 9 days reprogramming (LAP (N=6): day 6 - P=0.020, day 9 - P=0.948; CR2,3,4 (N=6): day 6 - P=0.004, day 9 - P=0.708). Interestingly, although C/EBPβ mutants with deletion in either CR3 or CR4 reprogrammed the primary B cells into myeloid cells (though ΔCR4 did it partially), the double deletion mutant ΔCR3,4 induced neither CD11b expression nor CD19 downregulation. This reveals the essential and redundant role of TAD CR3 and CR4 during B cell reprogramming and indicates that an important protein module in the TAD must be missing when CR3 and CR4 are simultaneously deleted. Surprisingly, the rescue experiments showed that CR2,3,4 but not CR3,4 (in combination with bZip) is the region that is sufficient and necessary to reprogram B cell progenitors into myeloid cells. In conclusion, our experiments with protein deletion mutants revealed that the capacity of C/EBPβ to reprogram B cells to myeloid cells resides in the TAD CR2,3,4 and, interestingly, both functions, downregulation of CD19 and upregulation of CD11b, depend on these TAD modules of C/EBPβ.

Comprehensive mass spectrometry data have shown that C/EBPβ is extensively post-translationally modified through threonine (T) and serine (S) phosphorylation, lysine (K) acetylation, mono- and dimethylation of arginine (R) residues and mono-, di- and trimethylation of K residues (Leutz et al., 2011) (Fig. 1.2). Therefore, a set of C/EBPβ mutants with substitutions in amino acid (AA) residues subjected to PTMs, where mutations were introduced in the TAD, RD or bZip region of WT C/EBPβ LAP*, was additionally tested. R residues in LAP* were mutated to alanine (A) (to eliminate potential methylation site while maintaining secondary structure), to leucine (L) (to mimic methylation) or to glutamine (Q) (to mimic citrulination). Additionally, our

mutant-set comprised K substitution mutants abrogating G9a K methylation sites (e.g. K39/K168A ((Pless et al., 2008) and our unpublished data)), and constructs with mutated SUMO attachment site or the binding site of the SUMO-conjugating enzyme UBC9 (Kowenz-Leutz and Leutz, unpublished data), both disrupting SUMOylation (e.g. K156A, K156A/E158A, K156R). CD11b induction was detected for all tested AA substitution mutants suggesting that these mutated residues, which are potential target sites of PTMs, are not sufficient to abrogate C/EBPβ reprogramming function (Fig. 3.3). This is not surprising, if one considers that deletion of CR1, 2, 5, 6, and 7, and even CR3 and 4 (when they were individually deleted) did not abolish reprogramming.

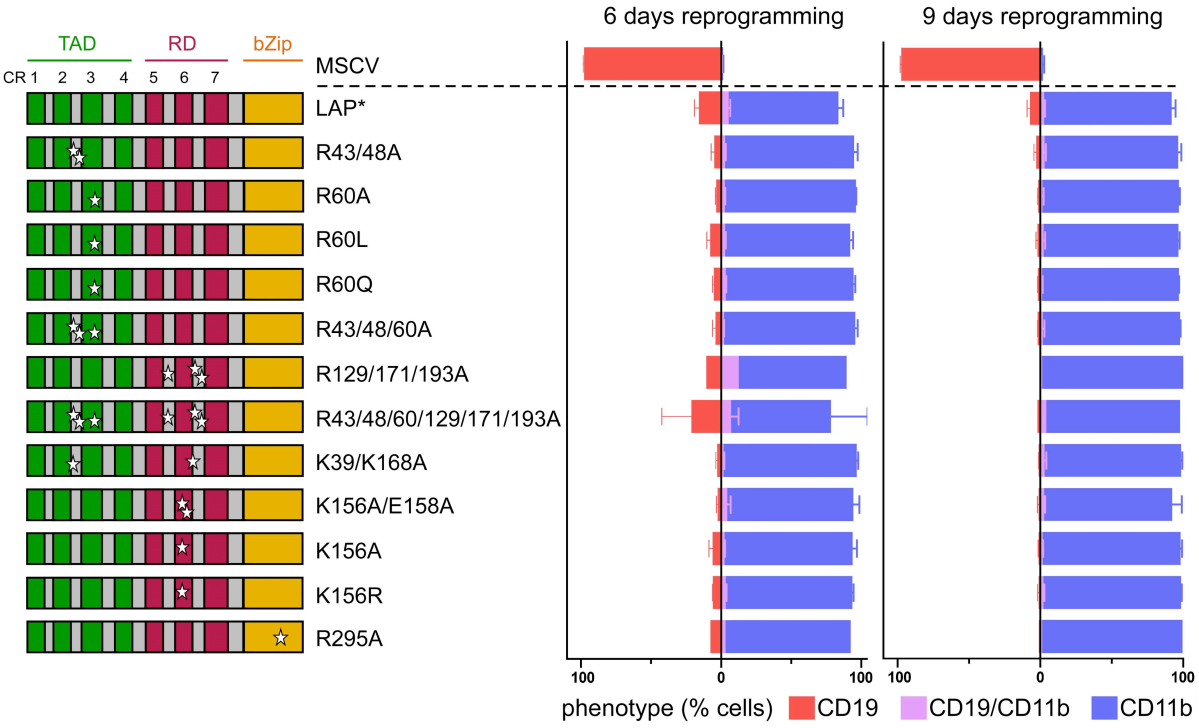


Fig. 3.3 Reprogramming of B cell progenitors by C/EBPβ PTM site mutants.
Schematic representation of the C/EBPβ AA substitution constructs (left panel) and percentage of cells infected with C/EBPβ LAP* and AA substitution mutants expressing the B cell marker CD19, the myeloid cell marker CD11b, or both markers simultaneously ($CD19^+$ $CD11^+$ cells) 6 days (middle panel) or 9 days (right panel) after the infection. Graphs represent mean ± SEM from GFP^+ (virus infected) gated cell population.

The expression and proper size of the C/EBPβ constructs was validated by western blot analysis of the packaging cell line (Fig. 3.4A). Additionally to IRES-GFP

fluorescence read-out, intracellular protein staining confirmed the expression of WT or mutant C/EBPβ proteins in the primary retrovirally infected B cells (Fig. 3.4B). All C/EBPβ reprogrammed CD11b$^+$ cells had comparable C/EBPβ ectopic expression levels, which were much higher than endogenous C/EBPβ expression in B cells (as determined in MSCV-infected cells), and even higher than the endogenous levels in WT BM derived macrophages.

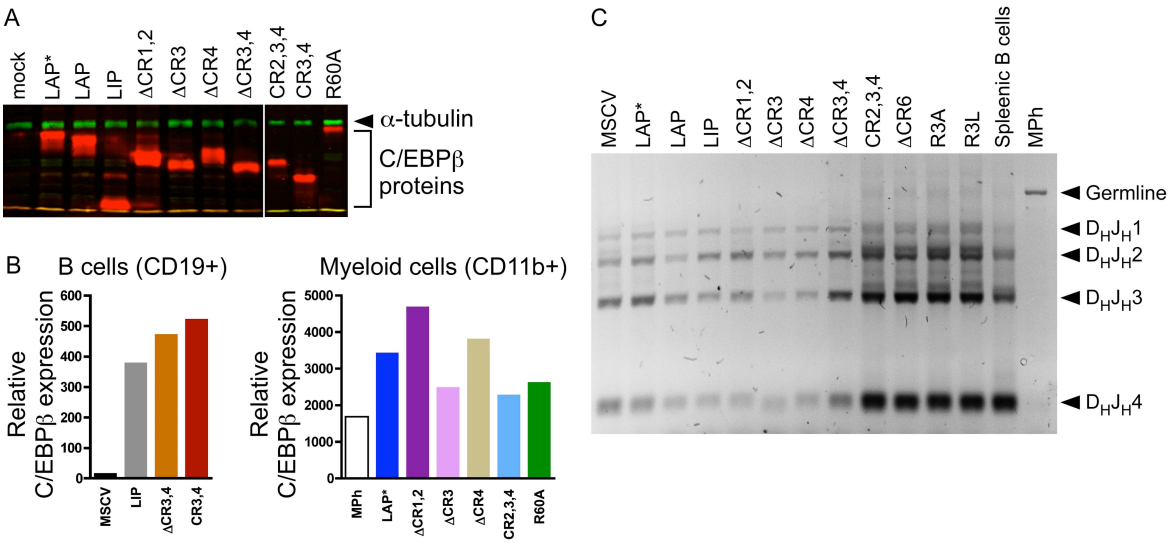


Fig. 3.4 C/EBPβ is overexpressed and IgH gene loci rearranged in the C/EBPβ reprogrammed myeloid cells.

A. Protein expression of the C/EBPβ WT and deletion constructs in the virus-packaging cell line Plat-E. The size of the proteins is according to the size of the deletions. B. Intracellular C/EBPβ protein staining in the reprogrammed cells. The relative C/EBPβ expression in the virus-infected cells was calculated as described in Materials and Methods. The endogenous C/EBPβ expression level in WT BM derived macrophages (MPh) was also assessed. The relative C/EBPβ expression values varied between the different experiments, however the tendencies were highly reproducible. C. PCR for D-J rearrangements in IgH locus. CD11b$^+$ reprogrammed myeloid cells and CD19$^+$ MSCV-, LIP- and ΔCR3,4-infected B cells were sorted 9 days after the infection and PCR for D-J rearrangements in the IgH locus was performed. Controls: WT BM derived macrophages (MPh) and splenic B cells. Data shown are representative of multiple experiments.

During B cell development rearrangements in the immunoglobulin (Ig) genes occur and this unique process allows B cell origin of the reprogrammed cells to be proven by Ig rearrangement PCR for D-J recombinations in the IgH locus (Ehlich et al., 1994; Xie et al., 2004). In control BM derived macrophages the IgH locus remained in germline configuration, whereas reprogrammed CD11b$^+$ cells showed the same

rearrangements as the splenic B cell control and the MSCV infected CD19$^+$ control cells, ruling out contamination with myeloid cell precursors (Fig. 3.4C).

3.2 C/EBPβ-mediated B cell reprogramming does not depend on the endogenous C/EBPβ

C/EBPβ deficient B cell progenitors were further tested to exclude the possibility that B cell to myeloid conversion occurred through an auto-regulatory loop by initial activation of endogenous C/EBPβ (Mink et al., 1999; Niehof et al., 2001). These data showed that, similarly to WT B cell progenitors, *C/EBPβ*$^{-/-}$ primary B cells could be reprogrammed to CD11b$^+$ myeloid cells by the ectopic expression of C/EBPβ, although the kinetics of reprogramming was somewhat accelerated in *C/EBPβ*$^{-/-}$ cells (Fig. 3.5A compared to Fig. 3.1). Hence, B cell to myeloid reprogramming by C/EBPβ does not depend on upregulation of the endogenous *C/EBPβ* gene. Interestingly, certain differences became apparent between the efficiency of LAP and LAP* to down-regulate CD19 in *C/EBPβ*$^{-/-}$ B cells (Fig. 3.5A, C). Both LAP and LAP* upregulated CD11b expression very effectively, however the extended isoform LAP* (which recruits the SWI/SNF complex) down-regulated CD19 expression more efficiently (similar differences between LAP and LAP* were also seen in some of the experiments with WT B cells, however they were not consistent). Furthermore, in *C/EBPβ*$^{-/-}$ B cell progenitors, the truncated isoform LIP showed some residual reprogramming capacity, which was not seen in WT cells. These discrepancies might be attributed to the lack of the endogenous C/EBPβ which may to some extend "buffer" the isoform specific effects.

It has been shown that during the reprogramming of B cells to macrophages by C/EBPα, the expression of the endogenous C/EBPα is not affected, whereas the endogenous C/EBPβ is upregulated (Bussmann et al., 2009). To test whether the lack of endogenous C/EBPβ might alter C/EBPα-mediated B cell reprogramming, primary B cell progenitors derived from WT or *C/EBPβ*$^{-/-}$ mice were compared. No difference in the reprogramming capacity of C/EBPα p42 long isoform was detected in WT and *C/EBPβ* deficient B cells (Fig. 3.5B). This suggests that, like C/EBPβ, C/EBPα-induced B cell reprogramming does not require endogenous C/EBPβ. Moreover, the observation that the truncated C/EBPα p30 isoform which lacks the C/EBPα TAD corresponding to C/EBPβ CR2,3,4, failed to reprogram B cells (Fig.

3.5B) further suggests that the major reprogramming functions of C/EBPs reside within their TADs.

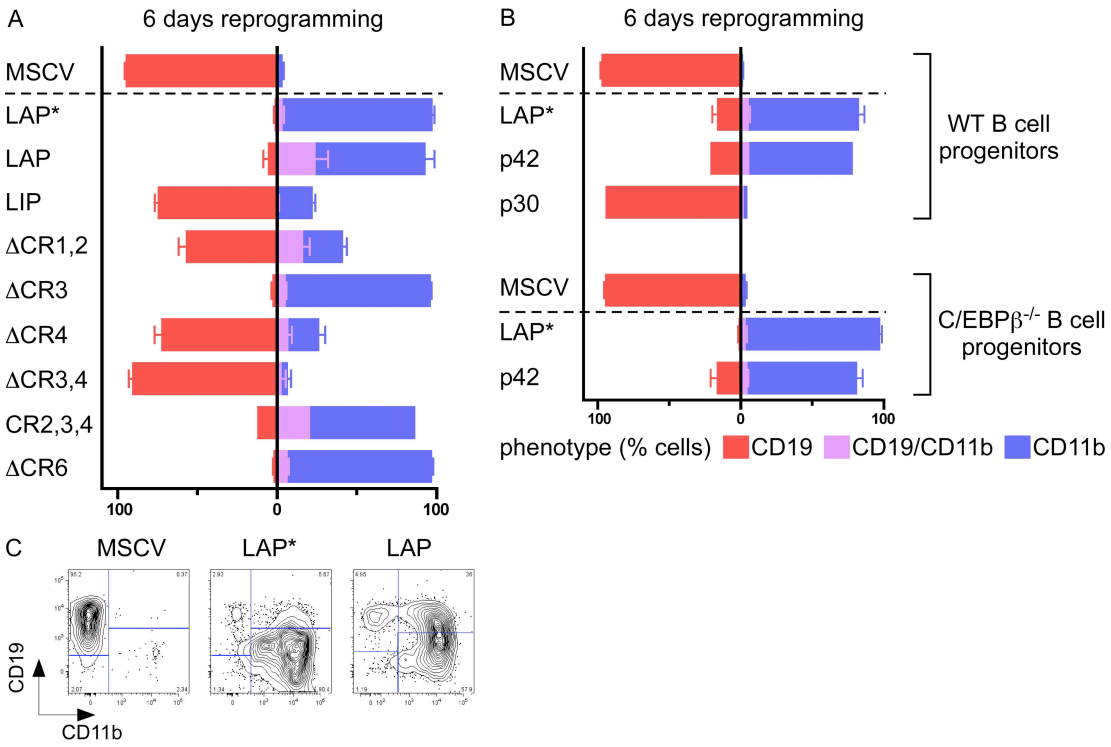


Fig. 3.5 C/EBPβ B cell reprogramming capacity does not depend on the presence of endogenous C/EBPβ.

A. Percentage of *C/EBPβ*$^{-/-}$ B cell progenitors infected with C/EBPβ WT and mutants expressing the B cell marker CD19 or the myeloid marker CD11b 6 days after the infection. Intermediates (CD19^{+} CD11^{+} cells) are also included. Graphs represent GFP^{+} (virus infected) gated cell population. Values represent mean ± SEM from two and more repeat experiments. B. Percentage of WT and *C/EBPβ*$^{-/-}$ B cell progenitors infected with WT C/EBPβ LAP* and WT C/EBPα p42 and p30 expressing the B cell marker CD19 or the myeloid marker CD11b 6 days after the infection. Intermediates (CD19^{+} CD11^{+} cells) are also included. Graphs represent GFP^{+} (virus infected) gated cell population. Values for *C/EBPβ*$^{-/-}$ B cell progenitors represent mean ± SEM from three repeat experiments. C. Representative FACS profiles of MSCV, LAP* and LAP infected *C/EBPβ*$^{-/-}$ B cell progenitors after 6 days reprogramming. FACS plots represent GFP^{+} (virus infected) gated cell population. Similar outcomes were obtained in three repeat experiments.

3.3 C/EBPβ B cell reprogramming and proliferation stimulating functions could be uncoupled

Previously, it has been shown that in contrast to C/EBPα, the percentage of cells infected with C/EBPβ markedly increases over time during B cell reprogramming (Xie

et al., 2004). As shown in Figure 3.6, the population of control vector MSCV infected cells remained fairly constant between day 6 and 9, whereas the long isoforms LAP*, LAP and mutants that supported reprogramming also increased cell numbers. Surprisingly, the percentage of the cells infected with the short isoform LIP decreased over time. Interestingly, the C/EBPβ mutants ΔCR3,4 and CR3,4 which failed to reprogram still induced proliferation (although P value was not statistically significant for CR3,4). This suggests that, unlike LIP, ΔCR3,4 and CR3,4 still retain capacity to induce cell proliferation. On the other hand, the PTM site mutants R60A (P=0.805) and R43/48A (P=0.652) did not induce higher proliferation of the reprogrammed cells compared to the B cells, proposing involvement of CR3 and the adjacent LCR between CR2 and CR3 in the proliferation control by C/EBPβ (Fig. 3.6 and data not shown). This suggests that C/EBPβ B cell reprogramming and proliferation-stimulating functions could be uncoupled. The uncoupling could be achieved either through deletions that abrogate the reprogramming, while keeping the proliferation-stimulatory function, or through PTM site mutations (in that case abrogating potential methylation sites) which preserve the reprogramming function, however abolish the stimulation of cell proliferation. However, proliferation ceased after 2 weeks *in vitro* culturing (data not shown) which might reflect the dual role of C/EBPβ in the regulation of hematopoietic cell proliferation, stimulation of the proliferation of B lymphoid progenitors and inhibition of myelomonocytic cell proliferation (Chen et al., 1997; Gutsch et al., 2011), or reaching the Hayflick limit (Shay and Wright, 2000).

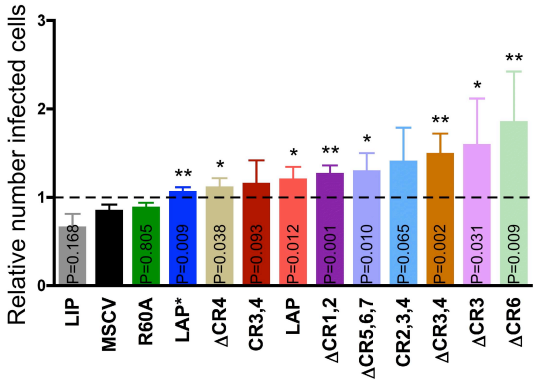


Fig. 3.6 Effect of C/EBPβ WT and mutants on cell proliferation.

Relative number of infected GFP$^+$ cells 9 days after the infection, calculated as a ratio between the percentage of GFP$^+$ cells on day 9 to the percentage of GFP$^+$ cells on day 6, where the percentage of GFP$^+$ cells on day 6 was taken as value "1". Values represent mean and SEM from different number of independent experiments. P values are all calculated versus MSCV. Number of repetitions: MSCV N=11, LIP - 4, R60A - 2, LAP* - 11, ΔCR4 - 4, CR3,4 - 2, LAP - 6, ΔCR1,2 - 5, ΔCR5,6,7 - 3, CR2,3,4 - 6, ΔCR3,4 - 5, ΔCR3 - 4, ΔCR6 - 4.

3.4 WT C/EBPβ and its mutants differentially activate the expression of key macrophage pro-inflammatory and anti-inflammatory genes

It has been shown that C/EBPα and β reprogrammed B cell progenitors display inflammatory macrophage phenotype (Xie et al., 2004). To analyze and compare how different C/EBPβ constructs contribute to the reprogrammed phenotype, we monitored the expression of several pro-inflammatory M1 and anti-inflammatory M2 macrophage genes, as well as key regulators of macrophage differentiation by NanoString technology (Table S1). We chose this technique because it is a highly reproducible method with low error rate (Fortina and Surrey, 2008; Geiss et al., 2008) and applicable to low amount of sample material, with which we had to deal due to the restricted numbers of reprogrammed primary cells. Additionally to C/EBPβ-reprogrammed cells, WT and *C/EBPβ*$^{-/-}$ BM derived macrophages were included, as it has been suggested that anti-inflammatory M2 properties rely on C/EBPβ and that M-CSF stimulated macrophages have similar gene expression patterns as M2 macrophages (Martinez et al., 2006; Ruffell et al., 2009).

Figure 3.7 and Table S2 show that many M1 (*Il1b*, *Il12b*, *Il12rb1*, *Nos2*, *Cxcl10*) and M2 genes (*Arg1*, *Msr1*, *Il13ra*, *Pparg*, *Chi3l3*) were upregulated during trans-differentiation and in macrophages from WT or *C/EBPβ*$^{-/-}$ animals as compared to B cell progenitors. Some genes like *Tnf*, *Il1b*, *Ccl2*, *Mmp9*, *Myd88*, *Il13ra*, *Msr1*, *Tgfb1*, *Mmp12*, *Pparg*, *Kdm6b*, *Chi3l3* and *Fcgr3* were expressed at similar levels in WT and *C/EBPβ* deficient macrophages, whereas, others, including *Il12b*, *Il12rb* and *Nos2* (all M1 genes) were upregulated in trans-differentiated cells but were neither expressed in WT nor in *C/EBPβ*$^{-/-}$ macrophages. However, differential gene expression between both genotypes was also identified, as *Mmp8*, *Mafb*, *Maf* and *Il4ra* demonstrated higher expression in WT as compared to *C/EBPβ*$^{-/-}$ macrophages. Interestingly, *Il10* was expressed in WT but not in *C/EBPβ*$^{-/-}$ macrophages, nor in the reprogrammed cells. On the other hand, *Arg1*, *Ccl22*, and *Cxcl9* (which was also not expressed in the C/EBPβ reprogrammed cells) were expressed only in *C/EBPβ*$^{-/-}$ but not in WT macrophages. These three genes, among which two code for chemokines, might be C/EBPβ repressed targets. Furthermore, in comparison to the C/EBPβ reprogrammed CD11b^{+} myeloid cells, BM derived macrophages expressed higher levels of M2 genes (e.g. *Msr1*, *Pparg* and *Il10*) and did not upregulate some M1 genes (e.g. *Il12b*, *Il12rb* and *Nos2*) which is in agreement with the finding that M-

CSF-derived macrophages and IL-4 stimulated macrophages, which undergo M2 polarization, are similar (Martinez et al., 2006). For example, *Pparg* upregulation in monocytes/macrophages has been associated with anti-inflammatory properties, suggesting the predominant anti-inflammatory nature of the BM M-CSF-derived macrophages (Arnold et al., 2007; Bouhlel et al., 2007). In our study, the relatively small differences in macrophage polarization specific gene expression between WT and *C/EBPβ*$^{-/-}$ BM derived macrophages might be due to the culture conditions (supplemented only with M-CSF), as major differences in the activation of many macrophage genes require stimulation with LPS/IFNγ (Gorgoni et al., 2002; Ruffell et al., 2009; Tanaka et al., 1995; Uematsu et al., 2007).

For the majority of macrophage polarization genes, WT C/EBPβ isoforms LAP* and/or LAP were the strongest gene activators, whereas ΔCR1,2 reprogrammed cells showed lower or lacked gene activation capacity for certain M1 genes (*Il12b*, *Nos2*, *Cxcl10* and *Mmp9*), M2 genes (*Arg1*, *Pparg*, *Mmp12* and *Chi3l3*) and the transcription factor *Maf*. The fact that ΔCR1,2 construct only weakly activated both M1 and M2 genes is consistent with the observation that it is also partially impaired in reprogramming. Comparison between LAP*, LAP and ΔCR1,2 activated genes revealed certain genes whose expression was dependent on CR1 and CR2. Macrophage polarization genes that were similarly deregulated by LAP and ΔCR1,2 compared to LAP* included *Mmp8*, *Mmp9*, *Mmp12*, *Kdm6b*, *Pparg* and *Chi3l3*, suggesting that recruitment of SWI/SNF chromatin remodeling complex might be a prerequisite for their activation (Kowenz-Leutz and Leutz, 1999; Kowenz-Leutz et al., 2010). Other genes (*Cxcl10*, *Arg1*, *Il4ra*, *Maf*) were upregulated by LAP but not by ΔCR1,2 suggesting that they require the presence of intact CR2. Furthermore, some genes (*Il1b*, *Cxcl10*, *Ccl2*, *Arg1*, *Il4ra* and *Maf*) were more highly activated by LAP than LAP*, which is in agreement with previous reports showing that LAP might be the stronger activator on certain genes (Eaton and Sealy, 2003; Lee et al., 1996). The complexity of variegated C/EBPβ CR functions in macrophage gene activation was further highlighted by the finding that the LAP* and C/EBPβ deletion mutants but not LAP activated *Mafb*, whereas LAP was the strongest activator of the *Maf* (*c-Maf*) gene probably due to a compensatory effect of the lack of *Mafb* expression in these cells (Aziz et al., 2006). Furthermore, in agreement with the reprogramming impairment, ΔCR4 failed to activate many macrophage genes (*Il12b*, *Nos2*, *Pparg* and *Arg1*) or induced their expression to a lower extend (*Cxcl10*), whereas ΔCR3

and ΔCR6 trans-differentiated cells showed gene expression pattern similar to LAP* (Fig. 3.7 and Table S2). Interestingly, the ΔCR6 protein which was previously shown to be constitutively repressed (Kowenz-Leutz et al., 1994) activated both M1 and M2 macrophage genes in the reprogrammed cells, supporting the idea that the RD is not a repressive structure in B cells.

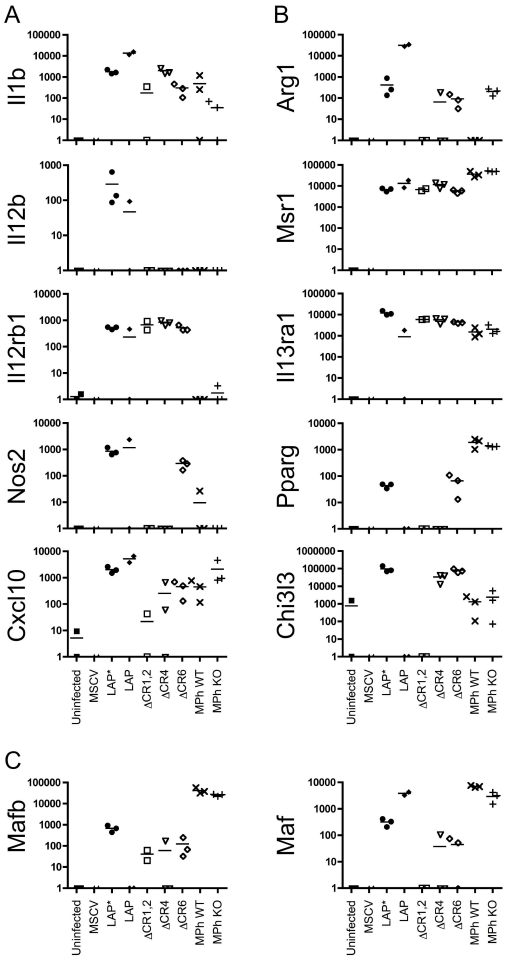


Fig. 3.7 Key macrophage genes are differentially expressed by reprogrammed C/EBPβ$^{-/-}$ B cell progenitors, WT and *C/EBPβ*$^{-/-}$ macrophages, as determined by Nanostring technology.

A. mRNA expression of pro-inflammatory M1 macrophage genes.

B. mRNA expression of anti-inflammatory M2 macrophage genes.

C. mRNA expression of the monocyte/macrophage transcription factors Mafb and Maf.

All graphs are presented as a logarithmic scale log10. Graphs represent mRNA counts after the background subtraction and normalization to three house-keeping genes (See Materials and Methods). Expression below the background level was set to value "1". MPh WT - WT BM derived macrophages; MPh KO - *C/EBPβ*$^{-/-}$ BM derived macrophages. Results represent expression profiles from three independent experiments.

Irrespective of these significant differences, the different C/EBPβ constructs simultaneously induced M1 and M2 genes in the trans-differentiated cells and did not show M1 or M2 bias. Some of these M1 genes (*Tnf*, *Nos2*, *Il1b* and *Il12b*) were already shown to be deregulated in *C/EBPβ* deficient macrophages upon LPS/IFNγ stimulation (*Tnf*, *Nos2*, *Il1b* are downregulated; *Il12b* is upregulated) (Gorgoni et al., 2002). Similarly, certain M2 genes (*Msr1*, *Il10*, *Il13ra*, *Arg1*) are dependent on CREB mediated C/EBPβ upregulation upon inflammatory stimuli (Ruffell et al., 2009). Therefore, the expression profile of the reprogrammed cells might resembles

LPS/IFNγ stimulated macrophages (Ruffell et al., 2009) with distinct pro- and anti-inflammatory genes showing C/EBPβ CR-dependent activation. The gene expression results also raised the possibility of the existence of heterogeneous trans-differentiated cell populations.

3.5 The C/EBPβ structure determines the trans-differentiation outcomes

It has been shown that high phagocytic activity is a hallmark on monocytes/macrophages, whereas DCs and granulocytes are characterized by a lower capacity of phagocytosis (Bakri et al., 2005; Drutman et al., 2012; Sunderkotter et al., 2004). Therefore, we decided to examine the potential functional heterogeneity in the cultures by performing an *in vitro* phagocytosis assay. Interestingly, this assay performed with C/EBPβ reprogrammed CD11b$^+$ cells revealed the presence of cells with high and low phagocytic activity (Fig. 3.8), suggesting heterogeneity among the reprogrammed cells. On the other hand, the MSCV control virus infected CD19$^+$ cells exhibited neglectable phagocytic activity, whereas WT BM derived macrophages showed homogenous phagocytic capacity.

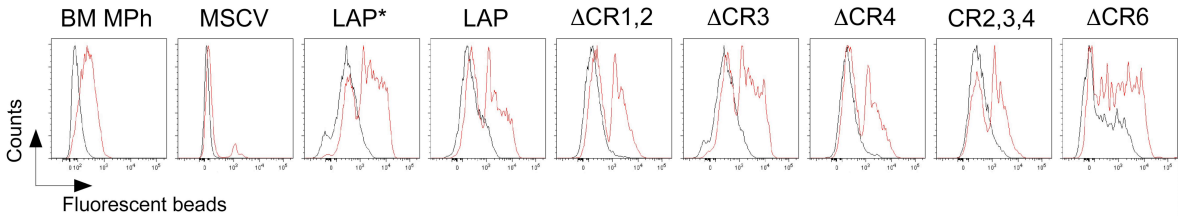


Fig. 3.8 Phagocytosis assay confirms the heterogeneity among C/EBPβ reprogrammed myeloid cells.

Assay was performed after 10 days *in vitro* reprogramming. Red line represents cells incubated with fluorescent latex beads and the black line represents the auto-fluorescence of the untreated samples. For MSCV-infected cells histograms represent GFP$^+$ CD19$^+$ gated population, whereas C/EBPβ-infected reprogrammed cells were gated on GFP$^+$ CD11b$^+$ cells. As positive controls for phagocytic capacity, BM derived macrophages were used. Similar outcomes were obtained in two repeat experiments.

Full length C/EBPβ and C/EBPα reprogram B cell progenitors to CD11b$^+$ F4/80$^+$ Gr-1$^+$ CD62L$^+$ inflammatory macrophages (Xie et al., 2004). To unravel heterogeneity of trans-differentiation outcomes by distinct C/EBPβ constructs, CD11b$^+$ cells were examined by flow cytometry for the expression of Gr-1/Ly-6C to distinguish between

inflammatory type monocytes/macrophages (CD11b$^+$ Gr-1/Ly-6C$^+$) and resident type monocytes/macrophages (CD11b$^+$ Gr-1/Ly-6C$^-$) (Arnold et al., 2007; Auffray et al., 2007; Geissmann et al., 2003; Nahrendorf et al., 2007). As shown in Table 3.1, C/EBPβ long WT isoforms and mutants reprogrammed B cell progenitors into two CD11b$^+$ subpopulations, in most cases resulting in high percentage of CD11b$^+$ Ly-6C$^+$ cells 6 days after the infection. However, 9 days after the infection, there was a significant decrease in the percentage of Ly-6C$^+$ myeloid cells and an increase in CD11b$^+$ Ly-6C$^-$ cells. The reduction of the percentage of Gr-1/Ly-6C$^+$ cells was not due to selective apoptosis, as no differences in the apoptotic cell frequency between CD11b$^+$ Gr-1$^-$ and CD11b$^+$ Gr-1$^+$ cells could be detected (Fig. 3.9). Accordingly, shift of CD11b$^+$ Ly-6C$^+$ to CD11b$^+$ Ly-6C$^-$ cell subset might be due to simultaneously occurring cell reprogramming and cell maturation processes (Drutman et al., 2012; Sunderkotter et al., 2004). Interestingly, constructs that did not downregulate Ly-6C by day 9 (e.g. ΔCR6) and such that did not considerably upregulate Ly-6C (e.g. LAP and CR2,3,4) or resulted in a small number of Ly-6C$^+$ cells by day 9 (e.g. ΔCR1,2) were also distinguished, suggesting that the activity of the individual protein modules in C/EBPβ might direct differentiation preferably towards inflammatory or resident monocyte/macrophage phenotypes. The reason for the relatively high percentage of

	LAP*	LAP	ΔCR1,2	ΔCR3	ΔCR4	CR2,3,4	ΔCR6
6 dpi [a)]	77 ± 1.7	39 ± 8.1	53 ± 6.9	78 ± 1.5	69 ± 1.8	18 ± 1.8	85 ± 1.5
9 dpi [a)]	51 ± 3.2	13 ± 2.4	19 ± 3.4	47 ± 1.1	39 ± 0.56	5.7 ± 2.0	71 ± 5.5
N [b)]	7	4	2	2	2	5	4
P value [c)]	<0.0001	0.021	0.047	0.003	0.004	0.002	0.046
Signifi-cance [d)]	Yes (***)	Yes (*)	Yes (*)	Yes (**)	Yes (**)	Yes (**)	Yes (*)

Table 3.1. Differential Ly-6C expression on the CD11b$^+$ cells reprogrammed by WT and mutant C/EBPβ.

[a)] Percentage of cells infected with WT and mutant C/EBPβ retroviral constructs expressing Ly-6C surface antigen 6 or 9 days after the infection. The total percentage of GFP$^+$ CD11b$^+$ cells was set to 100%. Values represent mean ± SEM; [b)] N - number of experiments; [c)] P values were calculated by unpaired t test 6 versus 9 days reprogramming; [d)] Significance was defines as P>0.05 - not significant, P=0.01-0.05 - significant (*), P=0.001-0.01 - very significant (**), P<0.001 - extremely significant (***).

Ly-6C$^+$ cells induced by ΔCR1,2 after 6 days reprogramming was probably due to the low percentage of CD11b$^+$ reprogrammed cells after this reprogramming period (Fig. 3.1) which perhaps led to an overestimation of CD11b$^+$ Ly-6C$^+$ cell outcome by this mutant on day 6, whereas, with the increase of the reprogramming efficiency on day 9, ΔCR1,2 gave similar outcome as LAP and CR2,3,4. Interestingly, all constructs that showed reduced Ly-6C$^+$ myeloid cell trans-differentiation outcome lacked CR1, suggesting its involvement in appointment of the resultant macrophage phenotype.

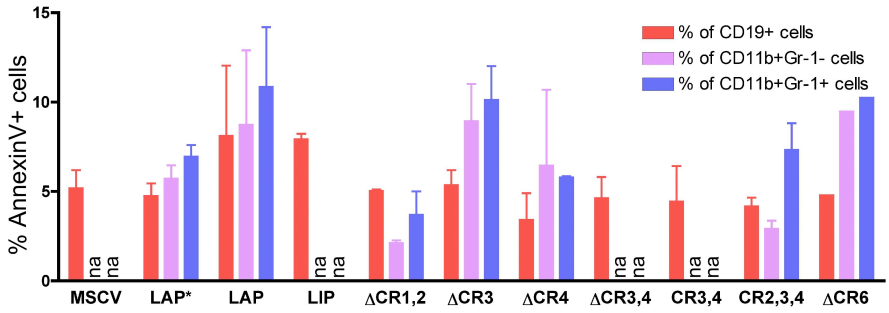


Fig. 3.9 Lack of significant difference in the frequency of the apoptotic cells between the subpopulations of cells ectopically expressing C/EBPβ.

Apoptosis assay based on AnnexinV staining and evaluated by FACS. Dead cells were excluded by DAPI staining and the apoptosis assessment was done after gating on the different GFP$^+$ cell populations (CD19$^+$, CD11b$^+$ Gr-1$^-$ and CD11b$^+$ Gr-1$^+$). na - no available cells with these surface characteristics. The graph represents data from four independent experiments.

CD11b is a marker of different types of myeloid cells, including monocytes/macrophages, granulocytes and DCs; furthermore Ly-6C is expressed not only on inflammatory monocytes/macrophages but also on granulocytes. Accordingly, additional surface markers were examined in order to distinguish in more detail between reprogrammed CD11b$^+$ myeloid cell phenotypes and to unravel potential trans-differentiation heterogeneity. It is known that granulocytes differ from monocytes/macrophages by the expression of M-CSFR on the latter and among M-CSFR$^+$ monocytes/macrophages Ly-6C/Gr-1 expression discriminates between inflammatory and resident monocytes/macrophages (Geissmann et al., 2003; Sunderkotter et al., 2004). As shown in Figure 3.10, CD11b$^+$ cells reprogrammed by full length and several C/EBPβ deletion constructs, simultaneously expressed Ly-6C (and Gr-1, data not shown) and M-CSFR surface markers, indicating an inflammatory monocyte/macrophage phenotype in accordance with published results (Xie et al.,

2004), however, we also identified Ly-6C$^-$ M-CSFR$^+$ resident type monocytes/macrophages and cells with granulocyte characteristics, namely Ly-6C$^+$ M-CSFR$^-$ cells (Geissmann et al., 2003; Sunderkotter et al., 2004), as well as CD11b$^+$ cells that did not upregulate either of these two markers (Ly-6C$^-$ M-CSFR$^-$ cells). The percentage of Ly-6C$^+$ M-CSFR$^+$ cells decreased between 6 and 9 days of reprogramming, whereas the percentage of Ly-6C$^-$ M-CSFR$^+$ cells increased (Fig. 3.10). This is most likely due to differentiation of inflammatory monocytes/macrophages into resident monocytes/macrophages, as shown before (Drutman et al., 2012; Sunderkotter et al., 2004). The remaining M-CSFR$^-$ Ly-6C$^+$ cells in the cultures expressed also Ly-6G, suggesting that this population corresponded to granulocytic differentiation (Fig. 3.11A) (Daley et al., 2008; Sasmono et al., 2007). However, no granulocytic differentiation was induced by LAP, CR2,3,4 and ΔCR1,2 and only a few inflammatory monocytes/macrophages were generated by LAP and CR2,3,4 (Fig. 3.10). In contrast, deletion of CR6 led to an increase of the Ly-6C$^+$ Ly-6G$^+$ M-CSFR$^-$ granulocytic population at the expense of the other populations (Fig. 3.11A).

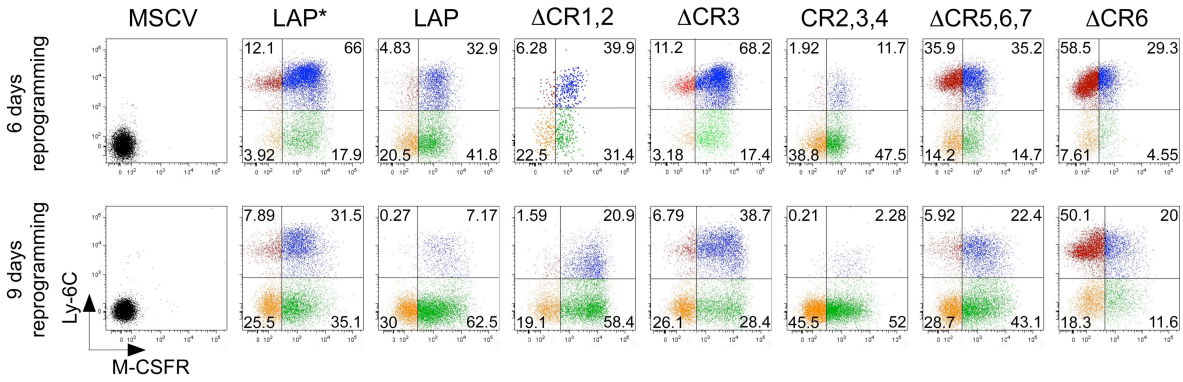


Fig. 3.10 Myeloid cell surface marker expression on the B cell progenitors reprogrammed by C/EBPβ WT isoforms and mutants after 6 and 9 days reprogramming.

Expression of Ly-6C and M-CSFR myeloid cell markers on the reprogrammed cells 6 days and 9 days after the infection. FACS plots represent GFP$^+$ (virus infected) CD11b$^+$ gated cell population. For MSCV-infected cells FACS plots represent virus infected GFP$^+$ CD19$^+$ cells. The myeloid cell marker staining was repeated in at least two independent experiments and similar results were obtained.

As the Ly-6C$^+$ M-CSFR$^+$ population represented inflammatory monocytes/macrophages, Ly-6C$^-$ M-CSFR$^+$ population - resident monocytes/macrophages, and Ly-6C$^+$ M-CSFR$^-$ cells granulocytic differentiation, we

were wondering about the rest of CD11b$^+$ cells that were Ly-6C$^-$ M-CSFR$^-$. CD11b is also a marker of cDCs, which are additionally characterized by high expression of CD11c, MHC-II and co-stimulatory molecules and are negative for M-CSFR expression (Geissmann et al., 2010). Further FACS analyses of the expression of

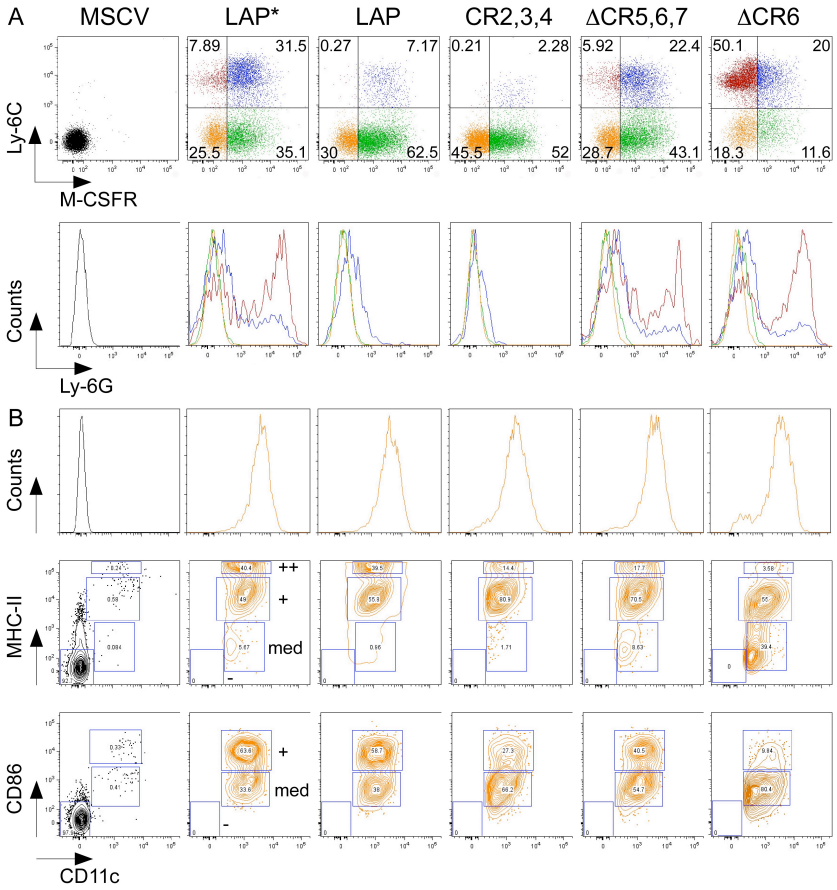


Fig. 3.11 Distinct myeloid cell surface marker expression on the B cell progenitors reprogrammed by C/EBPβ WT isoforms and deletion mutants.

A. Expression of Ly-6C, M-CSFR and Ly-6G myeloid cell markers on the reprogrammed cells 9 days after the infection. FACS plots and histograms represent GFP$^+$ (virus infected) CD11b$^+$ gated cell population. For MSCV-infected cells FACS plots and histograms represent virus infected GFP$^+$ CD19$^+$ cells. On the histogram for Ly-6G expression, lines are colour coded as the populations on the Ly-6C/M-CSFR FACS plot. B. Expression of the DC markers CD11c, MHC-II and CD86 on the reprogrammed Ly-6C$^-$ M-CSFR$^-$ cells 9 days after the infection. FACS plots and histograms represent GFP$^+$ (virus infected) CD11b$^+$ Ly-6C$^-$ M-CSFR$^-$ gated cell population (colour coded as the corresponding population on the Ly-6C/M-CSFR FACS plot in A). For MSCV-infected cells FACS plots and histograms represent virus infected GFP$^+$ CD19$^+$ cells. “++”, “+”, “med” and “-“ represent the expression levels of MHC-II and CD86 antigens. The cell marker staining was repeated in at least two independent experiments giving similar results.

CD11c, MHC-II and the co-stimulatory molecule CD86 showed that the reprogrammed CD11b^{+} Ly-6C^{-} M-CSFR^{-} cells were also characterized as CD11c^{+} MHC-II$^{+/++}$ CD86$^{+/med}$, phenotypic features of cDC (Fig. 3.11B and data not shown). We excluded pDCs as a trans- differentiation outcome, as their phenotype is defined as CD11b^{-} B220^{+} CD19^{-} Ly6C^{+} Ly6G^{-} CD115^{-} CD11c^{med} MHC-II^{lo} (Geissmann et al., 2010). The deletion mutant ΔCR6 not only induced lower DC outcome at expense of increased granulocytic trans-differentiation but these DCs had more immature phenotype suggested by their lower MHC-II and CD86 expression (Chow et al., 2002). Interestingly, Ly-6C^{+} M-CSFR^{+} inflammatory monocytes/macrophage population expressed low levels of DC markers or was negative for them, whereas the majority of Ly-6C^{-} M-CSFR^{+} resident monocyte/macrophage cells were CD11c^{+} MHC-II$^{+/med}$ CD86^{med} (Fig. 3.12 and data not shown). This intermediate phenotype between DCs and monocytes/macrophages might be associated with partial acquisition of DC markers from activated monocytes or initial differentiation step towards monocyte-derived DCs from the resident monocyte/macrophage population (Belz and Nutt, 2012; Drutman et al., 2012). Furthermore, the reprogrammed DCs

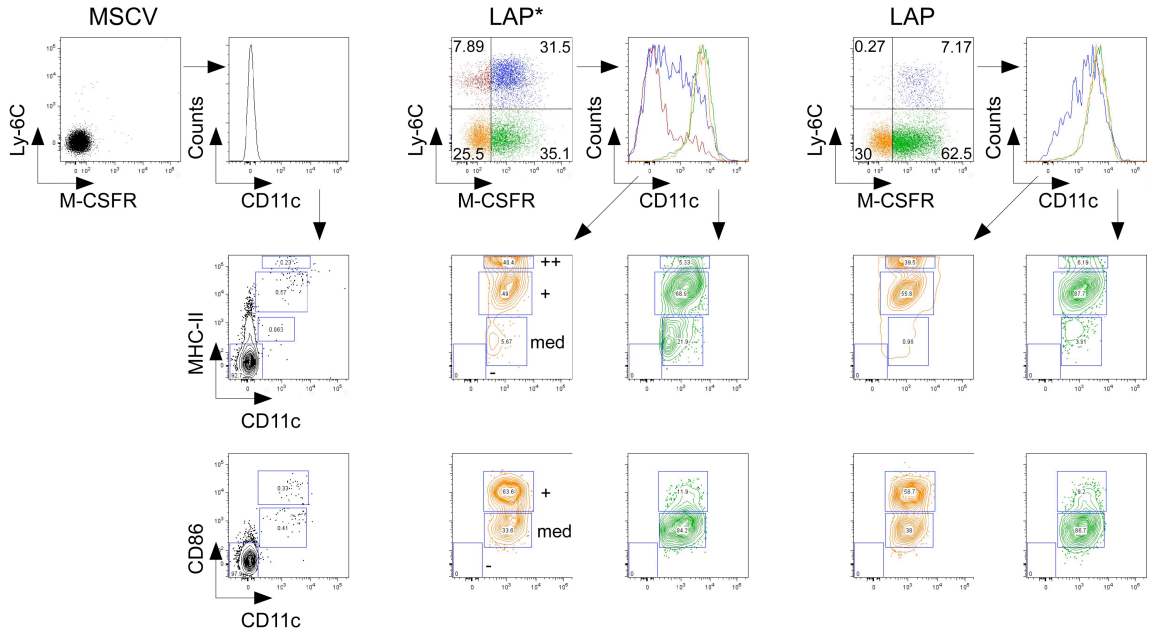


Fig. 3.12 DC markers are expressed also on the monocyte/macrophage populations.
Expression of the DC markers CD11c, MHC-II and CD86 on the reprogrammed cells 9 days after the infection. FACS plots and histograms represent GFP^{+} (virus infected) CD11b^{+} gated cell population and are colour coded as the corresponding populations on the Ly-6C/M-CSFR FACS plot. For MSCV-infected cells FACS plots and histograms represent virus infected GFP^{+} CD19^{+} cells.

and both monocyte/macrophage populations expressed F4/80 surface marker (data not shown), a marker of macrophages and subsets of DCs, also expressed on DCs derived through reprogramming of T cells by PU.1 (Laiosa et al., 2006b). In summary, structural alterations in C/EBPβ modified the reprogramming outcomes and based on the expression of surface molecules, $CD11b^+$ cells were reprogrammed to a heterogeneous population of cells with surface marker characteristics of inflammatory and resident monocytes/macrophages, granulocytes and cDCs.

Next, we determined the cell morphology of the reprogrammed cells by histological staining on cytospins of sorted GFP^+ virus infected cells 9 days after the infection. The control virus MSCV infected $CD19^+$ cells showed typical B cells morphology, whereas the morphological outcome of $CD11b^+$ cells reprogrammed by the WT or mutant C/EBPβ was very different depending on the construct (Fig. 3.13 and data not shown). Constructs that induced similar surface marker expression also induced morphologically similar trans-differentiated cells. For example, LAP*-, ΔCR3- and ΔCR5,6,7-reprogrammed cells, which based on the FACS analyses could be subdivided into Ly-$6C^+$ M-$CSFR^-$, Ly-$6C^+$ M-$CSFR^+$, Ly-$6C^-$ M-$CSFR^+$ and Ly-$6C^-$ M-$CSFR^-$ populations (Fig. 3.10), displayed morphologic characteristic of polymorphonuclear neutrophils, monocytes/DCs and macrophages, suggesting that deletion of CR3 and CR5,6,7 does not significantly affect the differentiation outcome. Other constructs, like CR2,3,4 and LAP, which generated Ly-$6C^-$ M-$CSFR^-$ and Ly-

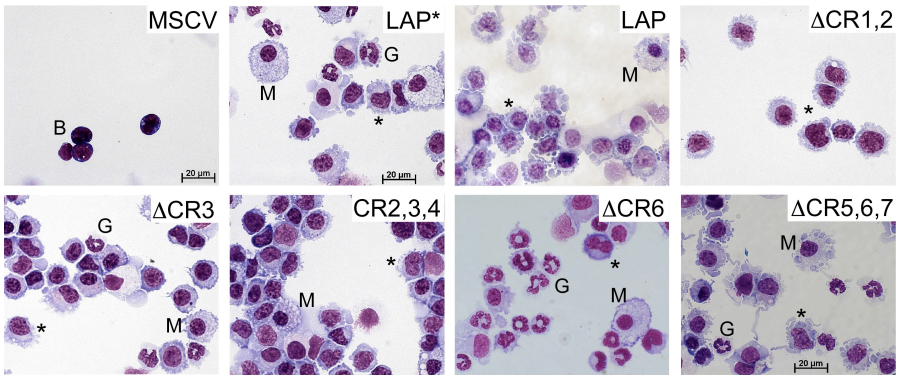


Fig. 3.13 Distinct cell morphology of C/EBPβ reprogrammed cells

Cytospins of the control virus MSCV infected $CD19^+$ cells and $CD11b^+$ cells reprogrammed by WT C/EBPβ and its deletion mutants sorted 9 days after the infection and stained with May-Grunwald-Giemsa. B - B cells, M - macrophages, G - neutrophil granulocytes, * - monocytes/DCs.

6C^{-} M-CSFR^{+} cells and a minor fraction of Ly-6C^{+} M-CSFR^{+} cells, led to monocyte/DC and macrophage differentiation, however no granulocytic differentiation could be detected, suggesting the requirement of CR1 for the granulocytic trans-differentiation outcome. Similarly, ΔCR1,2 failed to generate granulocytes but only less differentiated monocytes/DCs. Furthermore, in accordance to the FACS analyses, ΔCR6 reprogrammed cells had predominantly neutrophil granulocytic morphology. Hence, the surface marker expression reflected differences in the cell morphology of the reprogrammed cells, and depending on the function of discrete CRs in C/EBPβ abrogated or enhanced differentiation into distinct myeloid cell types. Except for the isoforms, deletions do not naturally occur in C/EBPβ, yet modular gene and chromatin regulatory functions of CRs have been noted before and found to be regulated by signaling dependent PTMs (Kowenz-Leutz et al., 2010; Leutz et al., 2011; Nerlov, 2008; Zahnow, 2009). The fact that recombination of C/EBPβ CRs affected myeloid trans-differentiation capacity therefore suggested regulation by PTMs. Accordingly, we used multiparameter flow cytometry to inspect more closely the myeloid reprogramming outcome by two C/EBPβ PTM site mutants which did not abrogate the reprogramming capacity of C/EBPβ (Fig. 3.3). As shown in Figure 3.14A, the AA substitution mutant K39/168A led to enhancement of the granulocytic

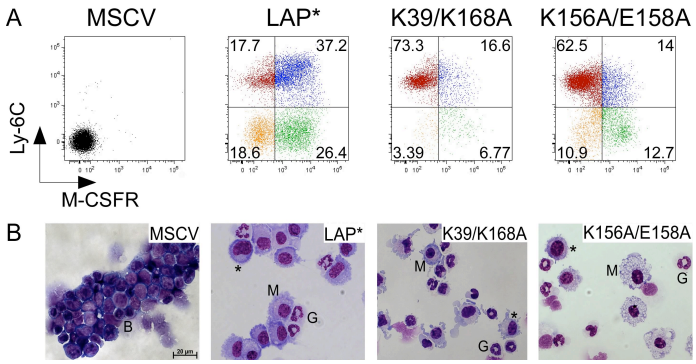


Fig. 3.14 C/EBPβ PTMs also affect the cell differentiation outcome from C/EBPβ-mediated B cell reprogramming.

A. Expression of Ly-6C and M-CSFR myeloid cell markers on the reprogrammed cells 9 days after the infection. FACS plots represent GFP^{+} (virus infected) CD11b^{+} gated cell population. For MSCV-infected cells FACS plots represent virus infected GFP^{+} CD19^{+} cells. B. Cytospins of the cells infected with WT C/EBPβ and its PTM site mutants sorted 9 days after the infection and stained with May-Grunwald-Giemsa. Experiments were repeated two to three times and similar results were obtained. B - B cells, M - macrophages, G - neutrophil granulocytes, * - monocytes/DCs.

differentiation, as compared to C/EBPβ WT LAP*. FACS data of cells reprogrammed by C/EBPβ K39/168A were confirmed by histological staining (Fig. 3.14B). Similarly, the majority of CD11b^{+} cells reprogrammed by the UBC9 binding and SUMOylation defective C/EBPβ mutant K156A/E158A were with granulocytic surface marker characteristics and morphology (Fig. 3.14A,B). Of note, residues K156, E158 and K168 are all located in CR6 or in the adjacent LCR and deletion of CR6 resulted in a dramatic increase in the granulocytic differentiation outcome (Fig. 3.11 and 3.13). In conclusion, PTMs on C/EBPβ, similarly to CR deletions, altered the reprogramming outcome, suggesting that C/EBPβ functions to direct distinct cell fates are adapted by signaling and PTMs. It is possible that some still unidentified PTMs might abrogate the granulocytic differentiation (one potential candidate is R3L (Kowenz-Leutz et al., 2010)), while others might distort DC or monocyte/macrophage differentiation.

3.6 *Irf8* deficiency affects neither C/EBPβ reprogramming efficiency nor the macrophage reprogramming outcome

It has been shown that IRF8 directs myeloid hematopoietic progenitor differentiation into macrophages at expense of granulocytes (Tamura et al., 2000). *Irf8*$^{-/-}$ B cells were used for the reprogramming experiments to answer the questions whether (i) *Irf8* deficient B cells could be reprogrammed, (ii) *Irf8* is essential for C/EBPβ-induced macrophage reprogramming outcome (iii) increased trans-differentiation into granulocytes occurs in absence of *Irf8*. Our data, based on downregulation of CD19 and upregulation of CD11b showed that after both 6 and 9 days reprogramming, *Irf8* deficient B cell progenitors were indistinguishable from their WT counterparts (Fig. 3.15). Detailed analyses of the myeloid cell differentiation spectrum showed that in the *Irf8* deficient genetic background after 6 days reprogramming, there was a dramatically impaired trans-differentiation into M-CSFR^{+} cells, including both Ly-6C^{+} and Ly-6C^{-} cells. However, after 9 days reprogramming this impairment was no more detectable and macrophage differentiation could be confirmed by both FACS analyses and cytospins (Fig. 3.15A,B). A two fold increase in the granulocytic outcome was seen in the *Irf8*$^{-/-}$ cells reprogrammed by LAP*, however this increase was not observed when CR2,3,4 mutant was used in accordance to our data from WT B cells (Fig. 3.11, 3.13 and 3.15). In summary, these data indicate that *Irf8* deficient B cells could be reprogrammed to myeloid cells by C/EBPβ similarly to WT

B cells and IRF8 presence is not essential for the macrophage reprogramming outcome, however, in the absence of IRF8 there is an increase in the granulocytic outcome.

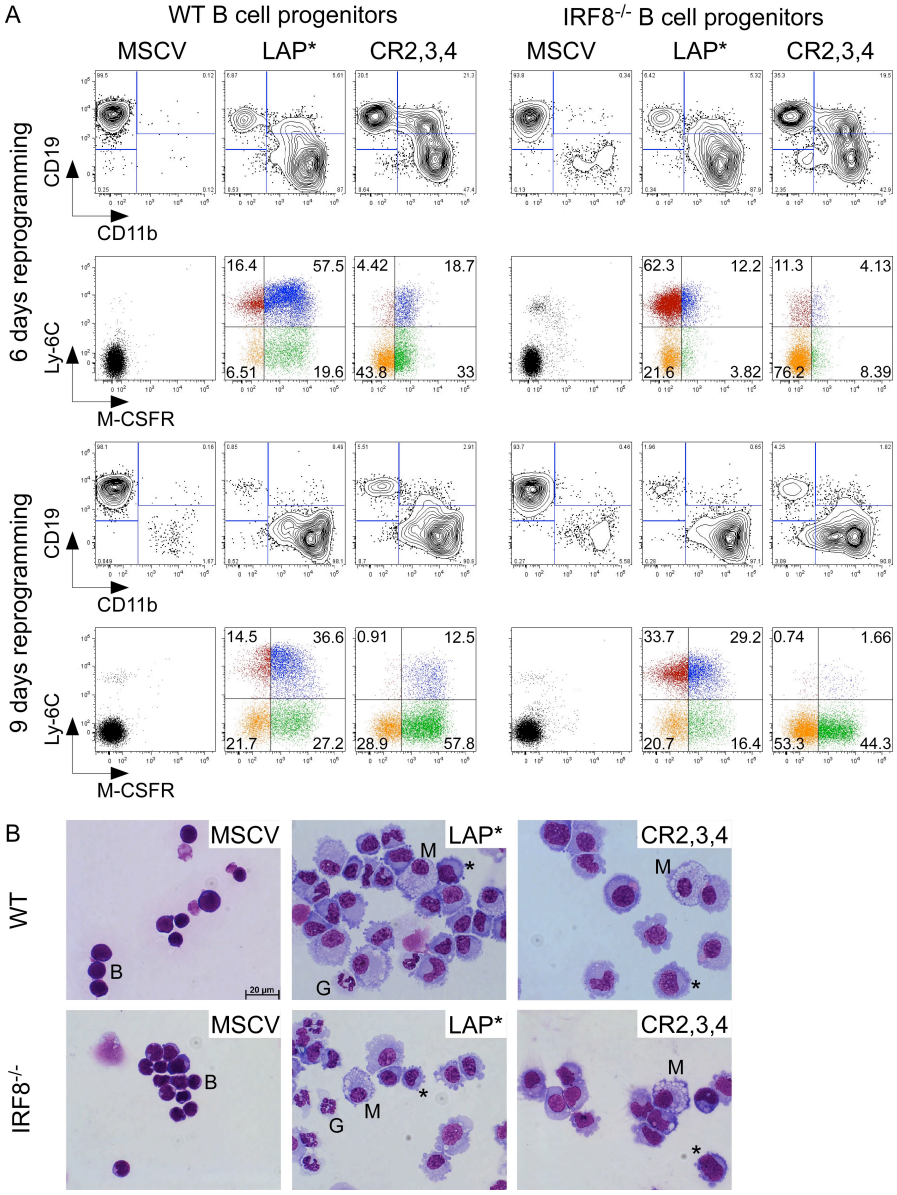


Fig. 3.15 C/EBPβ induced reprogramming and its outcomes are IRF8-independent processes.
A. FACS profiles of MSCV, LAP* and CR2,3,4 infected WT and *Irf8*$^{-/-}$ B cell progenitors after 6 and 9 days reprogramming. FACS plots represent GFP^{+} (virus infected) gated cell population. B. Cytospins of the WT and *Irf8*$^{-/-}$ reprogrammed cells infected with MSCV, LAP* and CR2,3,4, sorted 9 days after the infection and stained with May-Grunwald-Giemsa. B - B cells, M - macrophages, G - neutrophil granulocytes, * - monocytes/DCs.

3.7 C/EBPβ has a developmentally conserved essential function in G-CSF and GM-CSF signaling

It has been already shown that C/EBPβ deficiency in BM progenitors leads to impairment of G-CSF, GM-CSF and IL-3 cytokine-stimulated granulopoiesis (Akagi et al., 2008; Hirai et al., 2006). Furthermore, in our reprogramming system C/EBPβ induced granulocytic differentiation in the absence of G-CSF, suggested that C/EBPβ might be a key signaling molecule in cytokine signaling leading to granulocytic differentiation. We were wondering whether this is also true in another system like fetal liver (FL) hematopoietic progenitor cells. Although fetal and adult definitive hematopoiesis share many similarities, there are also substantial differences between them (He et al., 2011; Kikuchi and Kondo, 2006; Lessard et al., 2004; Mikkola and Orkin, 2006).

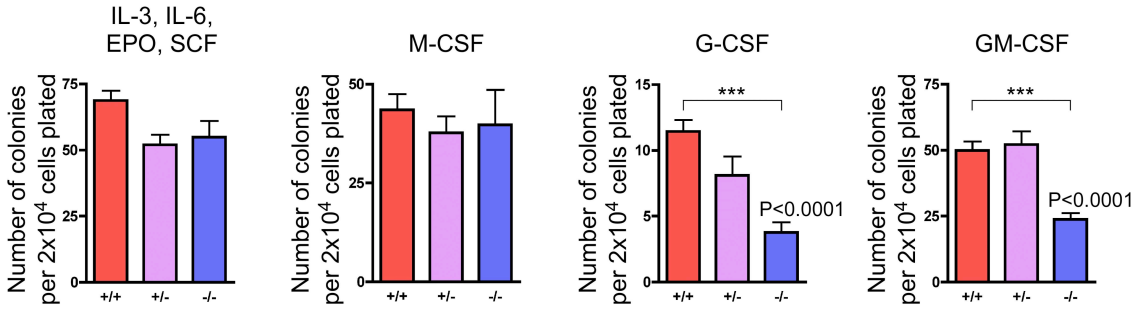


Fig. 3.16 G-CSF and GM-CSF responses are impaired in *C/EBPβ*$^{-/-}$ FL hematopoietic cells
Whole FL cells were isolated from *C/EBPβ*$^{+/+}$, $^{+/-}$ and $^{-/-}$ embryos at E14.5 dpc (N=3 for each genotype). 2x10^4 FL cells/well/1ml were cultured in methylcellulose supplemented with 50ng/ml SCF, 10ng/ml IL-3, 10ng/ml IL-6, 3 U/mL EPO, or with a single cytokine: 10ng/ml M-CSF, or 50ng/ml G-CSF, or 20ng/ml GM-CSF. Colonies were scored at day 7. Results represent the mean plus or minus SEM from three technical replicates for each of the three embryos per genotype.

To answer the question about the role of C/EBPβ in the context of fetal hematopoietic progenitors and stem cells specific transcription factor networks, whole FL cells were used as a source highly enriched in cycling HSCs and progenitors (Lessard et al., 2004; Mikkola and Orkin, 2006). *C/EBPβ*$^{+/+}$, $^{+/-}$ and $^{-/-}$ FL cells were analyzed by colony-forming unit (CFU) assay in methylcellulose supplemented with a distinct cytokine or a cytokine cocktail. The colony assay revealed that *C/EBPβ* deficiency did not significantly reduce the number of myeloid clonogenic cells when the FL cells

were cultured simultaneously with IL-3, IL-6, EPO, and SCF, or only with M-CSF (Fig. 3.16). Furthermore, in the mixed myeloid lineage cytokine cocktail no significant differences between the number of CFU-GEMM, CFU-GM, CFU-M, CFU-G or BFU-E were seen (data not shown). On the other hand, the number of colonies generated in the presence of either GM-CSF or G-CSF was significantly diminished in C/EBPβ deficient FL cells compared to WT cells. The heterozygous genotype was haplo-sufficient to rescue the WT phenotype and produced a significantly higher number of colonies in G-CSF and GM-CSF compared to *C/EBPβ*$^{-/-}$, whereas no significant differences between *C/EBPβ*$^{+/-}$ and *C/EBPβ*$^{+/+}$ were obtained for these cytokines. Hence, similarly to *C/EBPβ*$^{-/-}$ adult BM hematopoietic cells, FL cells displayed an impaired G-CSF and GM-CSF responses, suggesting a conserved key role of C/EBPβ in the signaling through these cytokines during FL and adult BM definitive hematopoiesis.

4. Discussion

Direct hematopoietic cell reprogramming or trans-differentiation of committed cells into another cell type can be achieved by stimulation through exogenously expressed cytokine receptors or targeted inactivation or enforced expression of lineage-determining transcription factors which play essential roles in the physiological cell differentiation (Borzillo et al., 1990; Bussmann et al., 2009; Cobaleda and Busslinger, 2008; Laiosa et al., 2006b; Nutt et al., 1999; Xie et al., 2004). Hence, a better understanding of how transcription factors determine lineage decisions will be instrumental in the elucidation of normal and aberrant differentiation processes and targeted regenerative therapies. Previously, we have shown that distinct functional modules of C/EBPβ are involved in interactions with the epigenetic machinery (Kowenz-Leutz and Leutz, 1999; Kowenz-Leutz et al., 2010; Mo et al., 2004; Pless et al., 2008). Using an experimental system where ectopical C/EBPβ expression reprograms primary B cell progenitors into myeloid cells, we show here that not only the long WT isoforms but also a variety of C/EBPβ deletion and PTM site mutants were capable to extinguish B cell commitment and activate a myeloid program (Fig.3.1, 3.2 and 3.3). The B cell origin of reprogrammed cells was confirmed by monitoring rearrangements in the IgH gene locus (Fig. 3.4C), which excluded the possibility that the emerging $CD11b^+$ cells were a result of outgrowth of myeloid cells due to contamination during the sorting procedure. Furthermore, lack of myeloid differentiation from the control vector MSCV-infected cells, or uninfected controls also confirmed the specificity of observed phenotypic changes (Fig. 3.1 and 3.2).

4.1 C/EBPβ isoforms and reprogramming

The long C/EBPβ isoforms LAP and LAP* differ from each other due to the usage of an alternative translation initiation site to generate a N-terminal 22 AA long region (CR1) which is present only in LAP* (Calkhoven et al., 2000). CR1 aids to differentially recruit SWI/SNF chromatin remodeling complexes and the LAP* specific CR1 functions are regulated by receptor tyrosine kinase/ mitogen-activated protein kinase (MAPK) mediated RD phosphorylation and methylation of R3 (Kowenz-Leutz and Leutz, 1999; Kowenz-Leutz et al., 2010). Accordingly, LAP and LAP* C/EBPβ long isoforms are reported to have some distinct functions in gene regulation.

Although LAP is the predominantly expressed C/EBPβ isoform and LAP and LAP* are equally efficient in plasmid reporter activation, subsets of chromosome-embedded genes in myeloid cells and in adipocytes are differentially regulated by LAP* and LAP (Kowenz-Leutz and Leutz, 1999; Kowenz-Leutz et al., 2010). In mice that lack the C/EBPβ LAP isoform but express the LAP* isoform, no impairment of intracellular bacteria killing is observed, whereas the induction of some C/EBPβ target genes in activated macrophages is altered (Uematsu et al., 2007), suggesting the existence of cellular functions that LAP* could not fulfill when LAP expression is missing. LAP and LAP* might also be subjected to distinct regulation by SUMOylation, which occurs only on LAP* and is involved in differential regulation of the cyclin D1 promoter (Eaton and Sealy, 2003). Our data showed that both LAP* and LAP were competent of B cell to myeloid trans-differentiation, meaning that both long isoforms possess the modules important for reprogramming (Fig. 3.1 and 3.2). However LAP* displayed faster kinetics, reflecting the fact that recruitment of chromatin-remodeling complexes might facilitate reprogramming, although they are not absolutely necessary for the process (Singhal et al., 2010). Similarly to LAP, C/EBPβ deletion mutants lacking CR1 (CR2,3,4 and ΔCR1,2) retained reprogramming competence, which was however significantly reduced compared to LAP*, supporting the idea that accomplishment of euchromatin state contributes to reprogramming efficiency.

4.2 C/EBPβ activation and minimal reprogramming region

C/EBPβ activity is auto-repressed and receptor tyrosine kinase - ras/MAPK signaling abrogates the auto-repression through phosphorylation, arginine and lysine (de-)methylation and protein conformational changes (Kowenz-Leutz et al., 1994; Lee et al., 2010a; Lee et al., 2010b; Mo et al., 2004; Williams et al., 1995). Distinct mutations in the regulatory sequence result in a fully de-repressed protein which could not be further activated by Ras/MAPK signaling (Kowenz-Leutz et al., 1994; Lee et al., 2010a). Deletion of CR5, CR7, or the whole RD (ΔCR5,6,7) of C/EBPβ results in enhanced transactivation potential compared to LAP*, whereas the deletion of CR6 results in a constitutively repressed protein with dominant negative function (Kowenz-Leutz et al., 1994; Williams et al., 1995). CR6 contains a highly conserved SUMOylation site which has been suggested to be involved in the regulation of

C/EBPβ repression/activation or protein subnuclear localization (Berberich-Siebelt et al., 2006; Eaton and Sealy, 2003; Kim et al., 2002; Leutz et al., 2011). SUMO family members are conjugated to C/EBPβ K173 in the human protein (K156 in the chicken protein) (Eaton and Sealy, 2003; Kim et al., 2002). Despite the fact that the SUMOylation site is present in CR6 in both long isoforms of the transcription factor, SUMO2/3 target only LAP* and mutation of SUMO-targeted K residue to A relieves the repression of the cyclin D1 promoter by LAP* without altering the protein subnuclear localization (Eaton and Sealy, 2003). In murine T cells, SUMOylation of C/EBPβ and its redistribution to a more pericentric heterochromatin interferes with the repression of *c-Myc* expression but has no effect on the activation of the *Il4* gene expression mediated by C/EBPβ (Berberich-Siebelt et al., 2006). Our reprogramming experiments showed that Δ CR5,6,7, ΔC R6 and the SUMO-site mutants K156A, K156A/E158A and K156R have similar to WT C/EBPβ LAP* capacity to reprogram B cell progenitors in terms of CD11b activation and downregulation of CD19 (Fig.3.1 and 3.3), suggesting that regulation of the transactivation potential of C/EBPβ in B cells is different from what has been observed in other cell types (Kowenz-Leutz et al., 1994). Furthermore, ΔCR6 activated both pro-inflammatory and anti-inflammatory macrophage genes in the reprogrammed cells (Fig. 3.7 and Table S2), supporting the idea that the RD does not serve as a repressive structure in B cells and during their reprogramming. That might be due to a lack of co-repressor in B cells and proposes a cell specific regulation of C/EBPβ functions. It should be noted that besides SUMOylation, methylation has been found to modify the SUMO attachment lysine residue (Leutz et al., 2011). It will be necessary to determine the exact modification patterns of the RD in different hematopoietic cells before one could set out to determine the modification dependent interactome, which will help to deepen our understanding of cell type specific C/EBPβ regulation.

Our group has already shown that the exchange of C/EBPβ DNA-binding and dimerization domain does not affect the functions of C/EBPβ TAD and RD (Kowenz-Leutz et al., 1994). Furthermore, exchanging the bZip domains between C/EBPα and C/EBPβ has pointed out that the TAD but not differential DNA-binding is responsible for certain preferential promoter activation by C/EBPα and C/EBPβ (Mink et al., 1999). In this context the mutant with exchanged leucine zipper CREB LZ is still effective in reprogramming B cells to myeloid cells (Fig. 3.1), meaning that C/EBPβ

reprogramming functions are not dependent on the native DNA-binding and dimerization C-terminal domain but on the N-terminus.

Mass spectrometry analyses have demonstrated that C/EBPβ is extensively post-translationally modified through R and K methylation (Leutz et al., 2011). Our previous data have shown that methylation has an inhibitory effect on C/EBPβ gene activation potential (Kowenz-Leutz et al., 2010; Leutz et al., 2011; Pless et al., 2008). In the current study we observed CD11b induction for all mutated R residues which we tested, suggesting that these single AA substitutions or their multiple combinations are not sufficient to disrupt C/EBPβ reprogramming function. That might not be unexpected as no abrogation of the cell reprogramming was accomplished after deleting CR1, 2, 5, 6 and 7, and even after individual deletion of CR3 or CR4 (Fig. 3.1 and 3.3).

Residue K39 in C/EBPβ serves as a target for either methylation by histone lysine methyltransferase G9a or acetylation by histone acetyltransferases p300 and P/CAF and these modifications regulate C/EBPβ transcriptional activity (Cesena et al., 2007; Cesena et al., 2008; Pless et al., 2008). It has been shown that acetylation of murine C/EBPβ LAP in K39 residue enhances the transactivation activity of C/EBPβ and exchanging this residue to A leads to a reduced transactivation on C/EBP responsive promoters (Cesena et al., 2007; Cesena et al., 2008). However, in contrast to these data, it has been demonstrated that the chicken C/EBPβ LAP* mutant K39A is resistant to methylation and thus repression by the G9a and has a hyperactive gene activation function (Pless et al., 2008). Here we showed that the mutant K39/K168A, bearing the mutation in the methylation/acetylation site K39A in combination with mutation in a second G9a targeted residue K168A in CR6 (our unpublished data), achieved myeloid reprogramming, meaning that reprogramming is independent of methylation/acetylation state of these residues.

Among the deletion mutants we tested, the mutant ΔCR3,4 was the one which, similarly to the short WT isoform LIP, lost C/EBPβ reprogramming function (Fig. 3.1 and 3.2). Interestingly, the important protein modules CR3 and CR4 showed redundancy, as the presence of any of them rescued C/EBPβ induced reprogramming. The observed redundant functions of C/EBPβ CR3 and CR4 during B cell reprogramming might be due to interaction with a protein partner which has binding sites to both regions, or differential interaction with two (or more) collaborating proteins. Moreover, CR3,4 in combination with the bZip was not

sufficient but depended on CR2 for B cell to myeloid cell reprogramming. (Fig.3.1), a discrepancy which might be due to improper protein conformation of C/EBPβ CR3,4. Similarly, dependence of the functionality of the repressive RD on the presence of CR6 has suggested the importance of CR6 for the maintenance of proper protein conformation (Kowenz-Leutz et al., 1994). Notably, CR3,4 exhibited severely impaired transactivating function (our unpublished data). Therefore, TAD CR2,3,4 emerged to be the structural module of C/EBPβ responsible for its reprogramming function.

CR2,3,4 represents the major TAD of C/EBPβ and entail several phosphorylation, methylation, and acetylation sites which are involved in multiple co-factor interactions. For example, C/EBPβ and p300 interact with each other through the N-terminus of C/EBPβ and p300 acts as C/EBPβ co-activator (Lee et al., 2010a; Mink et al., 1997). C/EBPα is shown to interact with TBP and TFIIB via a bipartite functional domain in the N-terminus and sequence homology to CR3 and CR4 in C/EBPβ are evident (Nerlov, 2008; Nerlov and Ziff, 1995). The histone lysine methyltransferase G9a methylates C/EBPβ and abrogates its transactivation potential and the interaction between C/EBPβ and G9a has been shown to occur through CR4 (Pless et al., 2008). C/EBPβ interacts through CR3 and CR4 with protein arginine methyltransferase 4 (PRMT4) which dimethylates R3 in CR1 leading to abrogation of the interaction with SWI/SNF and Mediator complexes (Kowenz-Leutz et al., 2010; Leutz et al., 2011). As discussed in the case of the RD, it will be necessary to unravel CR2,3,4 cell type specific PTMs and modification dependent interactome to mechanistically address the sequence of events involved in C/EBPβ mediated reprogramming.

C/EBPβ is naturally expressed in lymphoid cells and *C/EBPβ* deficient B cells display proliferation defects (Chen et al., 1997). This suggests silencing of C/EBPβ reprogramming function in normal lymphoid cells through cell type specific repressor complexes or their subunits that are specifically expressed in B lymphoid lineage but not in myeloid cells. For example, MTA3, a cell specific subunit of the co-repressor complex Mi-2/NuRD, has been implicated in cell-type specific gene repression and differentiation regulation (Fujita et al., 2004; Fujita et al., 2003). Furthermore, another component of this complex, Mi-2, could function independently of NuRD and has been found as a component of other alternative chromatin remodelers (Kunert and Brehm, 2009), suggesting that one protein subunit could be involved in different

complexes and hence different cellular functions. The mammalian SWI/SNF complex has different cell type specific subunit composition (Reisman et al., 2009; Wang et al., 1996). Interestingly, intrinsically repressed or activated C/EBPβ protein differentially interacts with either transcriptionally repressive or active Mediator complexes composed of alternative subunits and that depends on the methylation status of C/EBPβ R3 residue (Kowenz-Leutz et al., 2010; Mo et al., 2004). It is tempting to believe that PTMs on C/EBPβ might direct the differential interaction with its protein partners and that might restrict C/EBPβ activation in B lymphoid cells, however overexpression of C/EBPβ, like during experimental reprogramming, can overcome the repression by these B cell specific factors.

4.3 Reciprocal regulation of lymphoid and myeloid markers during the reprogramming and independence from endogenous C/EBPβ

Others groups have shown that reprogramming of B cells to myeloid cells is a gradual process, passing through an unstable bi-phenotypic intermediate state (Bussmann et al., 2009; Xie et al., 2004) and that was also valid for C/EBPβ long isoforms and mutants that we tested (Fig. 3.1, 3.2 and 3.3). These intermediate cells indicate that the lymphoid to myeloid conversion by C/EBPs does not involve retro-differentiation to hematopoietic progenitor and stem cells which then differentiate into the myeloid lineage, but rather takes a trans-differentiation ‘shortcut’ through bi-phenotypic intermediates (Di Tullio et al., 2011; Fukuchi et al., 2006). In this regard, C/EBPβ itself is most probably not sufficient to transform cells to leukemic ones and stabilize the bi-phenotypic state described in bi-phenotypic leukemias (Matutes et al., 1997; Zhao et al., 2009). We can speculate that a second mutation is needed to stabilize the intermediates induced by C/EBPβ in the process of reprogramming and this might then contribute to bi-phenotypic leukemic transformation. It is important to note that involvement of C/EBPβ mutations in human cancers is very rare and C/EBPβ tumorigenic function is connected mainly to deregulation of the isoform expression ratio or overexpression due to translocations to IgH locus (Akasaka et al., 2007; Vegesna et al., 2002; Zahnow, 2009). Furthermore, trans-differentiation of follicular lymphoma to histiocytic/DC sarcoma has been associated with high C/EBPβ expression, however in this case it is not clear whether this is a cause for or a consequence from the reprogramming (Feldman et al., 2008). Nevertheless, the

contribution of C/EBPβ induced reprogramming to leukemogenesis warrants further investigation.

It has been shown that B lymphoid to myeloid conversion by C/EBPs is dependent on synergy with endogenous PU.1, leading to the upregulation of CD11b, and repression of B lineage commitment factor Pax5 and downregulation of its target CD19 (Bussmann et al., 2009; Xie et al., 2004). None of the C/EBPβ mutant constructs differentially either upregulated CD11b or downregulated CD19, respectively Pax5. We also did not observe reprogramming through a CD19/CD11b double-negative state instead of a double-positive state (Fig. 3.2). This is in agreement with the published data that higher levels of C/EBPs are needed to inhibit Pax5 function than to activate the myeloid program in B cells (Bussmann et al., 2009). However, the kinetics of differential regulation of both CD19 and CD11b were delayed by C/EBPβ ΔCR1,2 and ΔCR4, as compared to LAP* (Fig. 3.1 and 3.2). This suggests that the C/EBPβ functions to antagonize Pax5 and to synergize with PU.1 both reside in TAD and might rely on SWI/SNF and Mediator recruitment (Kowenz-Leutz and Leutz, 1999; Kowenz-Leutz et al., 2010; Mo et al., 2004). Along these lines, the efficiency of LAP to downregulate CD19 as compared to LAP* was lower in trans-differentiating *C/EBPβ* deficient B cells (Fig. 3.5A, C). This suggests that both C/EBPβ functions to antagonize Pax5 and synergize with PU.1 in the activation of the myeloid program reside into TAD CR2,3,4, however, CR1 might further contribute to Pax5 repression. The LIP isoform, which acts as an inhibitor, not only failed to reprogram but also failed to downregulate CD19 expression, whereas it has been shown that lack of PU.1 disrupts CD11b upregulation by C/EBPs but not downregulation of CD19 (Bussmann et al., 2009; Xie et al., 2004). Accordingly, loss of B cell identity and activation of the myeloid program both require the C/EBP TAD but represent PU.1 independent and PU.1 dependent functions, respectively (Bussmann et al., 2009; Xie et al., 2004).

Since it has been shown that C/EBPs can reprogram B cell progenitors towards myeloid cell fate, the question about the dependence of C/EBPα and C/EBPβ reprogramming on the endogenous C/EBPs has been raised (Xie et al., 2004). In the process of reprogramming of a murine fetal pre B cell line to macrophages by C/EBPα, the family members C/EBPβ and C/EBPδ are upregulated and the dominant negative inhibitor C/EBPγ is faintly downregulated. Endogenous C/EBPα, which initially remains silent, becomes upregulated when reprogramming is already

completed (Bussmann et al., 2009). Our experiments with *C/EBPβ*$^{-/-}$ primary B cell progenitors showed that endogenous C/EBPβ was dispensable during the reprogramming process by ectopic expression of C/EBPβ and C/EBPα (Fig. 3.5) supporting the idea that reprogramming by C/EBPβ is not a simple transactivation of its own endogenous promoter but a more complex process.

4.4 C/EBPβ and cell proliferation control

Although uncoupling of CD19 downregulation from CD11b upregulation was not observed with any of the mutants examined, we achieved uncoupling of cell proliferation and trans-differentiation functions with some of the constructs. It has been observed that during B cell reprogramming the percentage of cells infected with C/EBPβ expands significantly over time compared to the control virus infected cells, whereas the percentage of C/EBPα infected cells decreases (Xie et al., 2004). Furthermore, C/EBPβ reprogrammed pre B cells exit the cell cycle with a delay compared to C/EBPα reprogrammed cells (Di Tullio and Graf, 2012). The current study confirms the proliferation stimulatory effect of C/EBPβ long isoforms as well as of C/EBPβ mutants during reprogramming. Interestingly, the deletion mutants ΔCR3,4 did not support reprogramming, yet still induced cell proliferation. PTM mutants with strong reprogramming potential (R60A, R43/48A; Fig. 3.6 and data not shown) did not support increased proliferation, suggesting that reprogramming function and proliferation control could be uncoupled. In this respect it is important to note that previously fat cell differentiation and proliferation arrest, induced by C/EBPα could be uncoupled, suggesting related underlying mechanisms of the C/EBP TADs in proliferation control (Muller et al., 1999). The observation that LIP did not support proliferation was surprising taking into account its proliferation enhancing function in mammary epithelial, breast cancer, and anaplastic large cell lymphoma cells (Gomis et al., 2006; Jundt et al., 2005; Zahnow, 2009). Furthermore, after partial hepatectomy *C/EBPβ*$^{\Delta uORF}$ hepatocytes (lacking only LIP expression) display reduced proliferation, later cell cycle entry and persistent repression of E2F target genes (Wethmar et al., 2010). However, the effect of C/EBPβ and its isoforms on the cell proliferation is highly context specific, because in some cases C/EBPβ displays growth-promoting activity and in others growth arrest (Johnson, 2005; Nerlov, 2007; Sebastian and Johnson, 2006). C/EBPβ deficiency leads to a reduced number of BM

B lymphocytes which have also decreased expansion in long-term culture (Chen et al., 1997). Under adipogenic differentiation conditions C/EBPβ deficient mouse embryonic fibroblasts (MEFs) do not undergo mitotic clonal expansion but can be rescued by ectopic expression of C/EBPβ LAP but not by LIP (Tang et al., 2003). Overexpression of a dominant negative C/EBP that antagonizes all C/EBP family members leads to a dramatic inhibition of proliferation of myeloid cell lines, whereas it does not have an effect on the proliferation of T cell and erythroid cell lines, suggesting cell type specific effects of C/EBPs on proliferation (Iwama et al., 2002). C/EBPβ functions in concert with RB/E2F pathway to inhibit proliferation of MEFs (Sebastian et al., 2005). Recently it has been shown that overexpression of LAP* in a monocytic cell line leads to a proliferation inhibition through RB/E2F pathway, as reduced phosphorylation of RB protein and reduced expression of c-Myc, E2F1 and cyclin D1 has been found (Gutsch et al., 2011). We can speculate that the reprogrammed cells, originating from B cells, still keep the hallmarks of B cell proliferation stimulation by C/EBPβ, whereas at later time points C/EBPβ myeloid growth inhibitory effects might become predominant (Fig. 3.6 and data not shown).

4.5 C/EBPβ structure and PTMs determine the myeloid cell type differentiation decisions

It has been suggested that C/EBPβ regulates the development of a variety of hematopoietic cell types in the myeloid compartment, including monocytes/macrophages, granulocytes and DCs (Akagi et al., 2008; Hirai et al., 2006; Iwama et al., 2002; Tanaka et al., 1995). C/EBPα and C/EBPβ may reprogram B cell progenitors to $CD11b^+$ $F4/80^+$ $Gr\text{-}1^+$ $CD62L^+$ inflammatory macrophages (Xie et al., 2004). Similarly, C/EBPα and C/EBPβ reprogram T cell progenitors to $CD11b^+$ $Gr\text{-}1^+$ $CD62L^+$ $CD11c^-$ MHC-II^- $F4/80^-$ inflammatory macrophages, whereas PU.1 reprograms the same cells to $CD11b^+$ $Gr\text{-}1^-$ $CD62L^-$ $CD11c^+$ MHC-II^+ $F4/80^{lo}$ myeloid DCs (Laiosa et al., 2006b). Ectopical expression of C/EBPβ or α in myb-ets transformed hematopoietic progenitors induces eosinophil differentiation, and C/EBPβ induces additionally myeloblast differentiation (Nerlov et al., 1998). C/EBPα expression in CLPs reprograms them into neutrophils and monocytes/macrophages (Iwasaki et al., 2006). However, C/EBPs inhibit DC differentiation, as it has been shown that C/EBPα and β promote granulocytic and monocyte/macrophage

differentiation at the expense of Langerhans DC differentiation (Iwama et al., 2002). However, that C/EBPβ may induce trans-differentiation of primary B cell progenitors into myeloid cell types other than macrophages has not been reported, even when the culture medium was supplied with G-CSF and GM-CSF, instead of M-CSF, although these reprogrammed macrophages expressed *G-CSFR* mRNA (Xie et al., 2004). The discrepancy with our results might be explained by the previous use of the C/EBPβ LAP isoform (which basically omits granulocytic trans-differentiation) (Xie et al., 2004) or differences in culturing conditions, such as prevention of cell contact between B cells and feeder cells in our system. Moreover, even though we supplemented the cultures only with M-CSF, the stromal cells might also have provided cytokines supporting granulocytic and DC differentiation. Furthermore, Flt-3L supplied to the cell culture can drive the differentiation of mouse hematopoietic BM progenitors into DCs *in vitro*. Interestingly, we observed typical DC clustering (Bakri et al., 2005; Manz et al., 2001) only in the cultures trans-differentiated by LAP and CR2,3,4 (data not shown). However, no differences in the cell differentiation outcome were seen when Flt-3L was omitted from the cultures (data not shown). Interestingly, studies have shown that M-CSF also has the capacity to support cDC and pDC differentiation *in vitro* in the absence of Flt-3L and *in vivo* in Flt-3L deficient mice, although M-CSF might not be as potent DC inducer as Flt-3L (Fancke et al., 2008).

Flt-3 and its ligand Flt-3L are key regulators of DC development and it has been shown that mTOR can act as a mediator of Flt-3L signaling in DCs. Adding mTOR inhibitor rapamycin into Flt-3L supplemented BMC cultures significantly reduces the number of all DC subsets, whereas rapamycin does not affected GM-CSF induced DC development in liquid culture (Sathaliyawala et al., 2010). In contrast, deletion of phosphatidylinositol 3-kinase (PI3K) - mTOR negative regulator *Pten* enhances Flt-3L induced DC development (Sathaliyawala et al., 2010). Other studies have shown that mTOR signaling is important for the survival and proper differentiation of the monocyte-derived DCs and short-time inhibition of mTOR reduces their immune-stimulatory features, whereas inhibition of mTOR does not negatively affect the cDCs differentiation (Haidinger et al., 2010). The inhibition of DC development by rapamycin treatment is in agreement with the published data that mTOR pathway inhibition influences C/EBPβ isoform ratio increasing the production of the long isoforms (Smink et al., 2009; Smink et al., 2012) and that C/EBPβ promotes

granulocytic and monocyte/macrophage differentiation from human CD34$^+$ cells and disrupts Langerhans DC differentiation (Iwama et al., 2002). Similarly, it has been shown that C/EBPβ direct target gene, the bZip transcription factor MafB (Smink et al., 2009), favors macrophage fate over DC differentiation (Bakri et al., 2005). Therefore, all current data support the idea that C/EBPβ long isoforms are acting as inhibitors of DC differentiation, whereas neutralization of the long isoforms through dominant negative C/EBP induces DC differentiation. Hence, the observed DC stimulatory function by C/EBPβ might be due to overcome DC repressive function through a counteracting protein, like for example the short isoform LIP or the negative regulator C/EBPγ, or due to a cell type specific effect, as all of the above mentioned experiments were performed in hematopoietic progenitor cells or myeloid precursors (Bakri et al., 2005; Iwama et al., 2002) but not in the context of the B cell specific transcription factor network.

Our data show that C/EBPβ isoforms and mutants may reprogram B cells to different myeloid cell lineages including granulocytes (Ly-6C$^+$ Ly-6G$^+$ M-CSFR$^–$), inflammatory monocytes/macrophages (Ly-6C$^+$ M-CSFR$^+$), resident monocytes/macrophages (Ly-6C$^–$ M-CSFR$^+$) and cDCs (Ly-6C$^–$ M-CSFR$^–$ CD11c$^+$ MHC-II$^{+/++}$ CD86$^{+/med}$) (Fig. 3.10 and 3.11). C/EBPβ has been shown to directly bind to CD11c gene regulatory elements (Lopez-Rodriguez et al., 1997), however the CD11c$^+$ DC phenotype does not seem a single marker activation, as those reprogrammed CD11c$^+$ cells also expressed very high amounts of MHC-II and CD86, which are typical features of DCs. However, within the myeloid cell pool, DCs and macrophages might share common markers, as they share a common precursor, which might make it difficult to assign them to one of these cell types (Geissmann et al., 2010). In accordance to that we detected high surface expression of CD11c and MHC-II and medium expression of CD86 on the population assigned as resident monocytes/macrophages (Ly-6C$^–$ M-CSFR$^+$), as well as low expression of CD11c, MHC-II and CD86 on the Ly-6C$^+$ M-CSFR$^+$ cells. Recently it has been shown that under sterile inflammatory conditions, both Ly-6C$^+$ and Ly-6C$^–$ spleen monocytes can acquire a DC surface phenotype with high expression of CD11c, MHC-II and co-stimulatory molecules, while simultaneously keeping monocytic lineage characteristics like M-CSFR expression, phagocytic function and poor antigen presentation, raising the possibility that activated monocytes might express DC surface markers without conversion into DCs (Drutman et al., 2012). Furthermore, under inflammatory conditions monocytes

can differentiate into monocyte-derived DCs, which similarly to cDCs express CD11c and MHC-II, however lose the expression of M-CSFR and Ly-6C (Belz and Nutt, 2012) suggesting that the reprogrammed resident monocyte/macrophage population could have undergone initial steps towards monocyte-derived DCs differentiation. Although the phagocytosis assay confirmed the presence of functional heterogeneity among the reprogrammed cells, other functional analyses (e.g. mixed lymphocyte reaction) will be required to prove the functionality of these CD11c$^+$ cells. Interestingly, lineage plasticity, shared lineage marker features and capacity of inter-conversion are observed not only between monocytes/macrophages and DCs but there is evidence that it might be a common characteristic of the cells from the myeloid lineage to gain properties of the other myeloid cell types. For example, Ly-6G$^+$ granulocytes can be trans-differentiated into F4/80$^+$ macrophage-like cells upon M-CSF stimulation (Sasmono et al., 2007). Furthermore, it has been shown that after incubation with T cells, mouse neutrophils can upregulate the expression of MHC-II and co-stimulatory molecules CD80 and CD86 and can present antigens to trigger T cell activation, suggesting that granulocytes can also acquire DC properties under certain conditions (Abi Abdallah et al., 2011).

It has been shown that Ly-6C/Gr-1$^+$ and Ly-6C/Gr-1$^-$ monocytes/macrophages differ in additional surface marker expression, gene expression profiles, biological functions and differentiation plasticity (Arnold et al., 2007; Auffray et al., 2007; Geissmann et al., 2003; Geissmann et al., 2010; Nahrendorf et al., 2007). For example, during skeletal muscle regeneration two monocyte/macrophage subsets sequentially emerge. Initially, Ly-6C$^+$ cells, which express higher levels of *Il1b* and *Tnf* mRNA and therefore exhibit pro-inflammatory properties, are recruited and they convert later on into anti-inflammatory Ly-6C$^-$ monocytes/macrophages, which express higher levels of *Tgfb1*, *Il10* and *Pparg* (Arnold et al., 2007). Furthermore, analysis of differentiation markers have shown that Ly-6C$^-$ anti-inflammatory monocytes/macrophages express CD11c and higher levels of F4/80 but are negative for the DC marker DEC-205, suggesting that Ly-6C$^-$ cells display high levels of macrophage differentiation markers compared to Ly-6C$^+$ cells (Arnold et al., 2007). In another study, it has been shown that Gr-1$^-$ monocytes might acquire M2 anti-inflammatory macrophage phenotype, whereas Gr-1$^+$ monocytes differentiate into DCs (Auffray et al., 2007). Furthermore, the healing myocardium sequentially mobilizes two monocyte subsets, Ly-6C^{hi} monocytes with inflammatory properties,

exhibiting higher MMP-2, -3, -9, and -13 proteinase activities and higher production of the pro-inflammatory cytokine TNF-α, whereas Ly-6C^{lo} monocytes promote healing and angiogenesis and express higher levels of vascular endothelial growth factor (VEGF) (Nahrendorf et al., 2007). Under inflammatory and noninflammatory conditions, both Gr-1^{hi} and Gr-1^{low} blood monocytes can give rise to lung DCs, however only Gr-1^{low} monocytes can differentiate into lung macrophages whereas Gr-1^{hi} monocytes gain this capability after conversion into Gr-1^{low} monocytes (Landsman et al., 2007). An additional layer of complexity is added by the finding that Listeria monocytogenes infection induces the recruitment of Ly-6C^{+} monocytes to the spleen where they differentiate into TNF-α and iNOS (Nos2)-producing Tip-DCs, providing further evidence for the relationship between monocyte/macrophages and DCs (Serbina et al., 2003).

As mentioned before, Ly-6C^{+} monocytes can convert into Ly-6C$^{–}$ monocytes (Arnold et al., 2007; Drutman et al., 2012; Sunderkotter et al., 2004) and in our experiments comparison between 6 and 9 days reprogramming suggested a similar scenario (Fig. 3.10). The observed decrease in the percentage of Ly-6C^{+} M-CSFR^{+} cells was not due to a higher apoptosis of the Ly-6C/Gr-1^{+} cells (Fig. 3.9). Furthermore, our data also suggested that at least some of the DC outcome might have resulted through differentiation from inflammatory or resident monocytes/macrophages, as DC representation also increased from day 6 to day 9. This is supported also by the observation that DC markers are expressed on the reprogrammed Ly-6C$^{–}$ M-CSFR^{+} resident monocytes/macrophages (Fig. 3.12). Another possibility is that the DC reprogramming had a delayed kinetics compared to monocyte/macrophage one, as on day 6 only a minority of the reprogrammed CD11b^{+} cells had DC characteristics, whereas on day 9 about quarter to half of the reprogrammed cells were CD11b^{+} Ly-6C$^{–}$ M-CSFR$^{–}$ CD11c^{+} which might have emerged from cells that were GFP^{+} CD11b$^{–}$ on day 6. One can also not exclude expansion of the DC population from day 6 to day 9 due to higher proliferation of these cells as compared to the other myeloid populations.

Comparison between surface marker expression, cell morphology and gene expression revealed that phenotypic differences seen between the cells reprogrammed by C/EBPβ WT or mutants correlated with differences in gene expression profiles (Fig. 3.7, 3.10, 3.11 and 3.13). For example, LAP* and ΔCR3 showed similar surface marker expression and morphology, additionally they had

similar macrophage gene expression pattern (Fig. 3.7 and Table S2). On the other hand, Δ CR4 and Δ CR1,2 failed to upregulate certain M1 and M2 genes, as compared to LAP* and ΔCR3, suggesting a more immature phenotype, impaired macrophage gene activation or alternative reprogramming into DCs. Comparison between the genes activated by LAP*, LAP and ΔCR1,2 nevertheless revealed that several macrophage polarization genes might depend on SWI/SNF recruitment. Notably, LAP and ΔCR1,2 were the constructs which, compared to LAP*, showed significantly lower induction of Gr-1/Ly-6C$^+$ cells (Table 3.1 and data not shown), suggesting that the differences seen in the surface marker expression reflected differences in macrophage gene activation. Interestingly, the granulocytic differentiation during B cell reprogramming was also dependent on the presence of CR1, as no neutrophil granulocytic differentiation was induced by LAP, CR2,3,4 and ΔCR1,2 (Fig. 3.10 and 3.13). This is in agreement with our previous results showing that activation of the neutrophil elastase gene in fibroblasts by C/EBPβ depends on its ability to recruit SWI/SNF which is, in turn, dependent on signal induced alteration in phosphorylation and arginine methylation (Kowenz-Leutz et al., 2010). Hence, our data support the notion that distinct CRs in C/EBPβ might be responsible for cell fate decisions by differential regulation of distinct genes.

As it was already emphasized, macrophages can exhibit a high level of plasticity and in response to environmental signals could be polarized into classically activated M1 and alternatively activated M2 macrophage phenotypes which have distinct biological functions and different gene expression profiles (Biswas and Mantovani, 2010; Mantovani et al., 2002; Solinas et al., 2009). Deregulation of many pro-inflammatory M1 genes upon LPS/IFNγ stimulation has been found in C/EBPβ deficient macrophages (*Il6*, *Tnf*, *Nos2* and *Il1b* are downregulated, whereas *Il12b* is upregulated) (Akagi et al., 2008; Gorgoni et al., 2002). Furthermore, it has been shown that inflammatory stimuli lead to upregulation of M1 and M2 genes in WT BM derived macrophages, however, prevention of CREB-mediated C/EBPβ upregulation affects activation of M2 genes (*Msr1*, *Il10*, *Il13ra* and *Arg1*), but not M1 genes (*Il1b*, *Il6*, *Il12b* and *Tnf*) (Ruffell et al., 2009). However, it is widely accepted that the concept of M1 and M2 polarized macrophage phenotypes might reflect the extremes of a spectrum of functional macrophage states and an operational oversimplification of various intermediate phenotypes (Biswas and Mantovani, 2010; Mantovani, 2008; Mosser and Edwards, 2008). Furthermore, macrophages can exhibit a remarkable

plasticity in response to environmental signals and change their functional state and macrophage plasticity is considered as a complication when investigating macrophage polarization (Biswas and Mantovani, 2010; Cassetta et al., 2011). For example, LPS stimulation of IFNγ treated BM derived macrophages enhances the expression of the M1 gene *Nos2* without inducing detectable expression of the M2 gene *Arg1*, whereas LPS stimulation of IL-4 treated BM derived macrophages induces simultaneously *Nos2* and *Arg1* expression, suggesting that the pattern of macrophage polarization gene expression might shift upon changes in the cytokine environment and that M1 and M2 macrophage polarization genes can be simultaneously activated upon treatment with a type 2 cytokine and LPS (Stout et al., 2005). Our mRNA expression analyses showed that C/EBPβ reprogrammed cells have upregulated simultaneously M1 and M2 genes and did not show M1 or M2 bias (Fig. 3.7 and Table S2). For example the already mentioned *Nos2* and *Arg1* were both upregulated by LAP*, ΔCR3 and ΔCR6, whereas ΔCR1,2 and ΔCR4 did not upregulate either of them. Interestingly, concomitant expression of *Nos2* and *Arg1* is a feature of regulatory macrophages, which are further characterized by high *Il10* expression and low *Il12* expression, however the lack of *Il10* expression on C/EBPβ reprogrammed cells argues against differentiation into regulatory macrophages in our experimental system (Cassetta et al., 2011; Mosser and Edwards, 2008).

Furthermore, the phenotype of the reprogrammed cells might resemble those of LPS/IFNγ stimulated macrophages, as it has been shown that inflammatory stimuli lead to upregulation of C/EBPβ and sequential activation of M1 and M2 genes in WT BM derived macrophages (Ruffell et al., 2009). This is in agreement with C/EBPβ protein expression data showing that C/EBPβ is overexpressed in the reprogrammed cells to levels higher than in M-CSF derived macrophages (Fig. 3.4B). However, we do not exclude the possibility that the reason we did not see a clear M1/M2 macrophage polarization might be due to the fact that our sorted $CD11b^+$ reprogrammed cells gave rise to mixed cell populations consisting not only of monocytes/macrophages, however also of DC-like cells and granulocytes, which can also produce pro- and anti-inflammatory cytokines (Mantovani et al., 2011; Shey et al., 2012). Furthermore, it has been shown that monocytes can differentiate under certain conditions *in vivo* and *in vitro* to a subtype of inflammatory DCs, the so-called Tip-DCs characterized by high TNF-α and NOS2 production (Serbina et al., 2003).

Interestingly, the genes coding for the two subunits of the IL-12 cytokine - *Il12b* (p40) and *Il12a* (p35) are shown to be both C/EBPβ target genes, however they have opposite regulation - *Il12a* is C/EBPβ activated gene, whereas *Il12b* is repressed by C/EBPβ (Gorgoni et al., 2002). Our gene expression data showed that *Il12b* M1 gene was consistently upregulated only by LAP* but not by the other constructs (LAP reprogrammed cells showed inconsistent *Il12b* expression levels). Experiments using macrophages from *C/EBPβ*$^{M20A/M20A}$ knock-in mice (lacking the expression of LAP) have shown that, similarly to *C/EBPβ*$^{-/-}$ genotype, these macrophages overexpress *Il12b*, suggesting that *Il12b* is a gene repressed by C/EBPβ LAP (Uematsu et al., 2007). However our gene expression data showed that *Il12b* was activated by C/EBPβ LAP* supporting the idea of the existence of non-overlapping and even opposite functions of the two C/EBPβ long isoforms.

Epigenetic studies have shown a relation between cytokine induced monocyte polarization, MAPK activation and H4 acetylation, proposing one potential mechanism for acquisition and maintenance of the macrophage polarization (Zhang et al., 2011). Other studies have shown the existence of a molecular pathway responsible for epigenetic regulation of murine key M2 polarization genes including Chitinase 3-like 3 (*Chi3l3*, also known as *Ym1*), *Retnla*, and *Arg1*. IL-4 stimulation leads to up-regulation of the lysine-specific demethylase 6B *Kdm6b* (*Jmjd3*), resulting in decreased H3K27 methylation (a modification correlated with transcriptional repression) at the promoters of M2 genes (Ishii et al., 2009). Furthermore, it has been found that *Kdm6b* is a gene essential for M2 but not M1 macrophage polarization in response to helminth infections (Satoh et al., 2010). Our data showed that *Kdm6b* was expressed by all C/EBPβ reprogrammed myeloid cells, although the highest expression levels were detected in LAP* reprogrammed cells (Table S2). Furthermore, M2 genes whose expression is dependent on *Kdm6b*, like *Arg1* and *Chi3l3*, were expressed by LAP*, ΔCR3, ΔCR4 and ΔCR6. Interestingly, LAP and ΔCR1,2 which showed lower activation of *Kdm6b* gene, as compared to LAP*, both failed to up-regulate *Chi3l3*, and *Arg1* was not expressed in ΔCR1,2 reprogrammed cells (Fig. 3.7 and Table S2). *Chi3l3* and *Arg1* are key M2 genes involved in extracellular matrix remodeling. *Arg1* codes for the enzyme Arginase1, which redirects the arginine metabolism towards polyamine synthesis and promotion of tissue regeneration (Biswas and Mantovani, 2012; Mosser and Edwards, 2008).

CHI3L3 is chitinase-like molecule, which lacks chitin-degrading activity, however it has carbohydrate and matrix-binding activity suggesting its role in matrix reorganization and tissue repair (Mosser and Edwards, 2008). Thus, our results support published data showing the dependence of certain M2 genes on *Kdm6b* chromatin modifying activity.

Interestingly, except the strong activation of *Maf* by LAP, all constructs upregulated *Mafb* and *Maf* to a lesser extend as seen in WT and *C/EBPβ*$^{-/-}$ macrophages. This is in agreement with the published data showing that M2 polarized macrophages (which are similar to M-CSF differentiated macrophages) have much higher expression of *Maf*, as compared to M1 macrophages (Martinez et al., 2006). Furthermore, high *Mafb* and *Maf* expression has been associated with M2 macrophage differentiation from Gr-1^{-} monocytes, whereas low *Mafb* and *Maf* expression and high PU.1 expression has been associated with inflammatory DC differentiation from Gr-1^{+} monocytes (Auffray et al., 2007). The low *Mafb* and *Maf* expression might explain the fact that we still observed cell proliferation between 6 and 9 days reprogramming (Fig. 3.6). However, our data do not support the idea that C/EBPβ reprogrammed cells are identical with unrestrictedly proliferating *Mafb*/*Maf* double-knock out macrophages (Aziz et al., 2009), as we failed to detect cell proliferation after 2 weeks of reprogramming in M-CSF supplemented medium (data not shown). Furthermore, during B cells to macrophage reprogramming by C/EBPα the expression of *Mafb* and *Maf* is also not upregulated (Bussmann et al., 2009), however, cell proliferation is inhibited, as compared to the controls and C/EBPβ reprogrammed cells (Xie et al., 2004). Furthermore, overexpression of LAP* in monocytic cells inhibits cell proliferation (Gutsch et al., 2011) probably through other, Maf-independent routes (Nerlov, 2007; Zaragoza et al., 2010).

It is widely accepted that the interplay including collaboration and antagonism between C/EBPs and other key myeloid transcription factors, in particular PU.1, is crucial for the cell fate decisions in the myeloid lineage and this might also determine the differential outcome from B cell to myeloid reprogramming by C/EBPβ (Bakri et al., 2005; Bussmann et al., 2009; Iwama et al., 2002; Laiosa et al., 2006b; Xie et al., 2004; Yeamans et al., 2007). In our reprogramming system those cells that accomplished high ectopical C/EBPβ expression, but medium/low PU.1 activation, might have become granulocytes. Interestingly, *C/EBPβ*$^{-/-}$ hematopoietic progenitor cells have impaired response to G-CSF and GM-CSF and *C/EBPβ* deficient

neutrophils exhibit increased apoptosis ((Akagi et al., 2008; Hirai et al., 2006) and Fig. 3.16). Additionally, SWI/SNF recruitment might be necessary for efficient granulocytic gene activation, as shown for the neutrophil elastase gene (Kowenz-Leutz et al., 2010). Other cells that achieved intermediate PU.1 activation, high ectopical C/EBPβ expression and high expression of the C/EBPβ target gene *Mafb* might have differentiated into macrophages (Bakri et al., 2005). Among the macrophage genes, M2 genes need higher C/EBPβ expression than M1 genes (Ruffell et al., 2009). In consensus with that it has been shown that *Mafb* and *Maf* are higher expressed in Gr-1^{-} monocytes differentiating into M2 macrophages than in Gr-1^{+} monocytes giving rice to DCs (Auffray et al., 2007). Finally, the cells that achieved relatively lower C/EBPβ expression and hence lower MafB expression, however expressed high levels of PU.1 might have differentiated into DCs (Bakri et al., 2005; Iwama et al., 2002). The low C/EBPβ/MafB expression levels might be accomplished physiologically during DC differentiation through the Flt-3 signaling for which it has been shown that it leads to mTOR activation (Sathaliyawala et al., 2010), resulting in a decrease of the translation of the long C/EBPβ isoforms whose directly activated target gene is *Mafb* (Smink et al., 2009). Furthermore, other factors, like IRF8 should be also considered as potential key players in the myeloid cell fate decisions (Rosenbauer and Tenen, 2007). The fact that we have obtained relatively few trans-differentiated cells however precluded a more detailed gene profiling study, which will also require separation of the different myeloid subpopulations by flow cytometric sorting. Nevertheless, it may be possible to generate sufficient cells with some of the C/EBPβ constructs that will enable us in the future to examine prevalence of key lineage determining factors, such as PU.1 and IRF8 or specific miRNA profiles (Baltimore et al., 2008).

Our data showed that *Irf8* deficiency affected the early steps of the reprogramming and M-CSFR^{+} cells were a minority (Fig. 3.15) in accordance to published data (Kallies et al., 2002). Later on, however, ectopical C/EBPβ expression overruled *Irf8* deficiency and little differences in the macrophage reprogramming outcome were observed. Of note, some differences in the trans-differentiation to Ly-6C^{+} M-CSFR^{+} monocytes/macrophages appeared, as previously shown (Becker et al., 2012) (Fig. 3.15A). It is important to mention that we were able to obtain BM derived macrophages from *Irf8*$^{-/-}$ BM cells and these macrophages showed no difference in the expression of M-CSFR compared to WT (data not shown). Although this is not a

quantitative result, as the macrophage differentiation might be due to an overgrowth of a small fraction of cells having the capacity to produce macrophages, this anyway suggests that *Irf8*$^{-/-}$ BM can give rise to M-CSFR^{+} macrophages. This is supported by recent finding that *Irf8* deficient mice still contain M-CSFR^{+} monocytes in BM, blood and spleen, although their numbers might be reduced, as compared to WT mice (Becker et al., 2012).

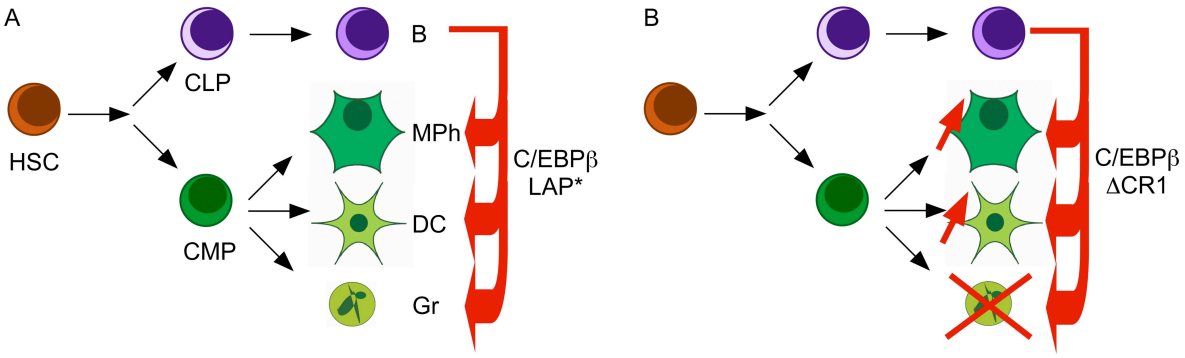


Fig. 4.1 The structure of C/EBPβ defines the outcome from B cell to myeloid lineage reprogramming

A. Simplified scheme of physiological hematopoietic lineage development (black arrows). Ectopical expression of C/EBPβ LAP* transcription factor in B cell progenitors leads to their reprogramming to monocytes/macrophages, DC and granulocytes (red arrows). B. Lack of CR1 in C/EBPβ (as in LAP, CR2,3,4 and ΔCR1,2) leads to abrogation of the granulocytic differentiation at the expense of an increase of the differentiation of the other myeloid cell types during B cell reprogramming. HSC – hematopoietic stem cells, CLP – common lymphoid progenitors, CMP – common myeloid progenitors, B – B lymphoid lineage, MPh – macrophages, DC – dendritic cells, Gr – neutrophil granulocytes.

Our current data showed that ectopic expression of LAP* led to the trans-differentiation of the primary B cell progenitors into granulocytes, inflammatory and resident monocytes/macrophages and DCs (Fig. 4.1). The other C/EBPβ long isoform LAP and the mutants CR2,3,4 and Δ CR1,2 lacking CR1 of TAD did not induce granulocytic differentiation, which suggests requirement for SWI/SNF recruitment for granulocyte cell differentiation program activation (Kowenz-Leutz et al., 2010). Other mutants like ΔCR6 showed increased granulocytic differentiation at the expense of the other populations (Fig. 3.11 and 3.13). However, not only certain protein regions (CRs) in C/EBPβ transcription factor were responsible for the instruction of distinct cell fates but we linked this observation to point mutations that

have been shown to reflect distinct signaling functions, such as modification by G9a (the K38/168A mutant), SUMOylation/methylation (as with the Δ CR6 mutant and previously with the methylation defective/mimicking R3A/R3L mutants involved in differential SWI/SNF recruitment (Kowenz-Leutz et al., 2010)) (Fig. 3.11, 3.13 and 3.14). These conclusions are supported by the observation that SUMOylation on C/EBPα is responsible for erythroid versus granulocytic lineage decisions during primitive hematopoiesis in zebrafish and C/EBPα in hypoSUMOylated state exhibits enhanced inhibition of GATA1 transcriptional activity and skews progenitor cell differentiation towards granulopoiesis (Yuan et al., 2011). Similarly, it has been shown that phosphorylation or phosphomimetic mutation in CR2 in C/EBPα inhibits granulocytic differentiation from retinoic acid treated bi-potential myeloid cell line, however the monocytic differentiation is not blocked, suggesting that phosphorylation on C/EBPα does not generally restrain differentiation but rather diverts cell fate decisions (Ross et al., 2004). Knockdown of SUMO conjugating enzyme UBC9 leads to impairment of adipocyte differentiation (Cignarelli et al., 2010). Therefore, PTMs could be a potential mechanism how C/EBPβ accomplishes differentiation to alternative myeloid cell types like monocyte/macrophage, granulocyte or DCs.

4.6 C/EBPβ, cytokine signaling and granulocytic differentiation

The exact role of C/EBPβ in G-CSF and GM-CSF signaling is not completely clarified, however, current evidence suggests involvement of C/EBPβ in the cytokine signaling leading to granulocytic differentiation. On one hand, C/EBPβ binding sites are found in the regulatory regions of genes coding for G-CSF and the receptors G-CSFR, GM-CSFR and M-CSFR (Tsukada et al., 2011). It has been shown that lack of all C/EBPβ isoforms or LAP deficiency leads to decreased G-CSF production from LPS-stimulated *C/EBPβ*$^{-/-}$ and *C/EBPβ*$^{M20A/M20A}$ (lacking LAP isoform) macrophages compared to WT macrophages (Tanaka et al., 1995; Uematsu et al., 2007). However, *M-CSF* and *GM-CSF* mRNA expression is not affected in *C/EBPβ* deficient macrophages upon inflammatory stimuli (Gorgoni et al., 2002; Tanaka et al., 1995). On the other hand, C/EBPβ deficiency in BM hematopoietic cells leads to impaired G-CSF and GM-CSF responses (Akagi et al., 2008; Hirai et al., 2006). However no difference in *G-CSFR* and *GM-CSFR* mRNA in *C/EBPβ*$^{-/-}$ and WT total BMC has

been seen (Hirai et al., 2006). Furthermore, *C/EBPβ*$^{-/-}$ neutrophils display an increased apoptosis compared to WT neutrophils (Akagi et al., 2008).

G-CSF is an essential cytokine for the growth and production of neutrophil granulocytes and their precursors and it signals through a homodimeric cytokine receptor G-CSFR, which leads to activation of Jak2 and/or Jak3 and subsequent phosphorylation and activation of STAT3. G-CSF signaling might also lead to activation of other pathways, like PI3K - AKT (also known as Protein Kinase B, PKB), or Ras-MAPK (Baker et al., 2007). However, despite the impaired G-CSF responses, no difference in STAT3 phosphorylation has been seen between *C/EBPβ*$^{+/-}$ and *C/EBPβ*$^{-/-}$ BMC stimulated with G-CSF (Akagi et al., 2008).

C/EBPβ is an auto-repressed transcription factor whose N- and C-terminus physically interact. Signaling through Ras-MAPK pathway leads to phosphorylation of C/EBPβ on an evolutionary conserved MAPK consensus site in RD CR7 and this phosphorylation results in protein activation (Kowenz-Leutz et al., 1994). Glycogen synthase kinase-3β (GSK-3β) has also been shown to phosphorylate C/EBPβ and to modify its functionality (Tsukada et al., 2011; Zahnow, 2009). One can hypothesize that G-CSF and/or GM-CSF signaling might, through Ras-MAPK pathway, or regulation of GSK-3β activity, or other signaling pathway, lead to acquirement of PTMs on C/EBPβ which might change its activity on target genes. These PTMs on C/EBPβ might include not only phosphorylation but also protein methylation, a PTM associated with a negative impact on C/EBPβ target gene activation (Kowenz-Leutz et al., 2010; Leutz et al., 2011; Pless et al., 2008). For example methylation-mimicking mutant of LAP* – R3L - shows inability to activate neutrophil elastase gene in fibroblasts and to induce adipocyte differentiation (Kowenz-Leutz et al., 2010). Furthermore, a crosstalk between C/EBPβ PTMs has been shown, as Ras/MAPK phosphorylation of C/EBPβ CR7 abrogates the interaction with PRMT4, the methyltransferase which methylates R3 (Kowenz-Leutz et al., 2010).

It has been shown that fetal and adult definitive hematopoiesis share common characteristics, however they also differ. For example fetal and adult HSC have phenotypical and functional differences: fetal HSCs are largely cycling, they undergo symmetric cell divisions and have an increased long-term repopulation compared to adult BM HSCs when transplanted into irradiated recipients (Harrison et al., 1997; Lessard et al., 2004). Fetal and adult HSC have a distinct transcriptional regulation and different surface marker profiles and FL hematopoiesis, in contrast to adult

hematopoiesis, involves mainly erythropoiesis and myelopoiesis, while lymphopoiesis is reduced (He et al., 2011; Lessard et al., 2004; Mikkola and Orkin, 2006). Furthermore, *Il7ra*$^{-/-}$ fetal HSCs but not adult BM HSC are capable of differentiation into B cells, suggesting that IL-7 is dispensable for FL B cell development (Kikuchi and Kondo, 2006). These altogether underline the necessity of a detailed characterization of the fetal hematopoiesis regulation and comparison between FL and BM definitive hematopoiesis.

Comparison between G-CSF and GM-CSF responses of FL and adult BM hematopoietic progenitor cells showed a developmentally conserved function of C/EBPβ in cytokine induced granulocytic differentiation. FL cells, similarly to their BM counterparts, showed impaired G-CSF and GM-CSF responses (Fig. 3.16). Although we did not go into any further details to analyze the molecular basis of the dependence of G-CSF and GM-CSF responses of FL cells on the presence of C/EBPβ, our current data and data from other labs point out that C/EBPβ is a crucial factor in granulocyte differentiation and possibly a key molecule in G-CSF and GM-CSF signaling (Akagi et al., 2008; Hirai et al., 2006; Jones et al., 2002). Similar function has been proposed for PU.1 in TNF-α and GM-CSF-induced DC differentiation in liquid culture (Bakri et al., 2005; Iwama et al., 2002). Interestingly, it has already been shown that C/EBPβ is a crucial intracellular mediator of IL-7 signaling and its deficiency leads to impaired proliferative responses of BM B lymphocyte progenitors to IL-7 (Chen et al., 1997).

Results presented here also show that the C/EBPβ structure and PTMs determined the outcome of B cell to myeloid cell reprogramming, revealing that some C/EBPβ mutants completely abrogated neutrophil granulocytic differentiation, while others increased the granulocytic outcome of the reprogramming. Interestingly, our data related the granulocytic differentiation to C/EBPβ CR1 (Fig. 4.1), suggesting dependence of the granulocytic differentiation on SWI/SNF recruitment (Kowenz-Leutz and Leutz, 1999) and potentially linking G-CSF and GM-CSF signaling to chromatin remodeling. One can speculate that in myeloid progenitor cells, granulocytes, macrophages and DCs C/EBPβ might have a different PTM pattern which determines the cell fate decisions through changing the activation state, interactome or cellular localization of C/EBPβ. This hypothesis should be examined through obtaining C/EBPβ protein from highly purified by FACS sorting GMP, granulocytic, monocytic, macrophage and DC populations and subsequent mass

spectrometric analyses. These data will complement the current data obtained through the reprogramming system and will shed light on the variety of mechanisms involved in regulation of C/EBPβ functions.

4.7 Concluding remarks and future directions

Cell fate decisions are defined by synergistic and/or antagonistic action of transcription factors, as well as their dosage and temporal sequence of expression (DeKoter and Singh, 2000; Hsu et al., 2006; Iwasaki et al., 2006; Laiosa et al., 2006a). Studies have also shown distinct roles of different protein isoforms of C/EBPs (Bedi et al., 2009; Smink et al., 2009; Uematsu et al., 2007; Wethmar et al., 2010). Using B cell lineage to myeloid lineage trans-differentiation approach, we were able to show that TAD CR2,3,4 is the essential protein module for C/EBPβ reprogramming function, responsible for both its B cell extinguishing and myeloid fate activating function. Interestingly, C/EBPβ structural and PTM site mutations led to different reprogramming outcomes, suggesting a potential mechanism for accomplishment of alternative myeloid cell fates by C/EBPβ.

C/EBPβ function in the macrophage differentiation, polarization and functionality is well known (Gorgoni et al., 2002; Gutsch et al., 2011; Ruffell et al., 2009; Tanaka et al., 1995). Our data also bring the attention to an often underestimated, however important function of C/EBPβ during granulopoiesis. Despite the fact that C/EBPα is considered to be the master regulator of granulopoiesis, the importance of C/EBPβ during granulocytic differentiation should not be neglected (Hirai et al., 2006; Jones et al., 2002; Zhang et al., 1997) and this is also supported by our data (Fig. 3.11, 3.13 and 3.16). Furthermore, in contrast to previous studies (Bakri et al., 2005; Iwama et al., 2002), our data suggest that C/EBPβ can act as a positive regulator of DC differentiation. One possible reason for these discrepancies might be that different set of transcription factors are expressed in B cells versus BM progenitors and that C/EBPβ might take advantage of the lymphoid branch to generate DCs. Nevertheless, a detailed understanding of the regulation of DC differentiation will provide novel tools for manipulation of DCs in new cancer immune-therapies and vaccination strategies.

Our data point out interesting questions about the potential differences between the myeloid cell subtypes in mice lacking or expressing a single C/EBPβ isoform, like

C/EBPβ^{LIP} and *C/EBPβ*$^{\Delta uORF}$ genotypes, and their comparison to *C/EBPβ*$^{-/-}$ and WT mice. Analyses of the expression of macrophage polarization genes in these *C/EBPβ* knock-in and *C/EBPβ* deficient macrophages are also planned. Another important question is the one concerning C/EBPβ interacting partners during the reprogramming. Mass spectrometry analyses of CR2,3,4 binding partners might help selecting for candidate proteins whose knock-down could be investigated in order to see whether reprogramming depends on them. Another important question is whether we will be able to find PTMs and their enzymes that might abrogate C/EBPβ reprogramming potential. Analysis of C/EBPα PTM site mutants in the B-to-myeloid cell reprogramming system is also of a current interest. Last but not least, uncovering differential C/EBPβ PTM profile in distinct hematopoietic cell types *in vivo* might help to deepen our knowledge of the regulation of cell fate decision by C/EBPβ.

Many lines of evidence suggest that C/EBPs may be involved in multivalent binding or assembly of regulatory complexes and link signaling to epigenetic regulation. Similarly to the “histone code”, the extensive PTM pattern seen on C/EBPβ suggests that multiple modifications of a transcription factors might be pivotal for integration of extracellular signals, gene expression and determination of diverse cell fate decisions (Leutz et al., 2011; Nerlov, 2008). Therefore, acquisition of signal dependent PTMs might explain how C/EBPβ accomplishes differentiation to alternative myeloid cell types.

Supplement

Table S1

Selected genes whose expression was analyzed in the reprogrammed cells and BM derived macrophages by NanoString technology.

	Gene symbol	Target ID	Full name
M1 genes	*Tnf* (*Tnfa*)	NM_013693.1	tumor necrosis factor
	Il1b	NM_008361.3	interleukin 1 beta
	Il6	NM_031168.1	interleukin 6
	Il12b	NM_008352.1	interleukin 12b
	Il12rb1	NM_008353.2	interleukin 12 receptor, beta 1
	Ccr7	NM_007719.2	chemokine (C-C motif) receptor 7
	Cxcl9	NM_008599.2	chemokine (C-X-C motif) ligand 9
	Cxcl10	NM_021274.1	chemokine (C-X-C motif) ligand 10
	Ccl2 (*Mcp-1*)	NM_011333.3	chemokine (C-C motif) ligand 2
	Mmp8	NM_008611.4	matrix metallopeptidase 8
	Mmp9	NM_013599.2	matrix metallopeptidase 9
	Nos2	NM_010927.3	nitric oxide synthase 2, inducible
M2 genes	*Il10*	NM_010548.1	interleukin 10
	Arg1	NM_007482.3	arginase, liver
	Il13ra1	NM_133990.4	interleukin 13 receptor, alpha 1
	Msr1	NM_031195.2	macrophage scavenger receptor 1
	Tgfb1	NM_011577.1	transforming growth factor, beta 1
	Il4ra	NM_001008700.3	interleukin 4 receptor, alpha
	Alox15	NM_009660.3	arachidonate 15-lipoxygenase
	Mmp12	NM_008605.3	matrix metallopeptidase 12
	Pparg	NM_011146.1	peroxisome proliferator activated receptor gamma
	Ccl22	NM_009137.2	chemokine (C-C motif) ligand 22
	Kdm6b (*Jmjd3*)	NM_001017426.1	lysine (K)-specific demethylase 6B
	Chi3l3 (*Ym1*)	NM_009892.1	chitinase 3-like 3
	Fcgr3	NM_010188.5	Fc receptor, IgG, low affinity III
Others	*Mafb*	NM_010658.2	v-maf musculoaponeurotic fibrosarcoma oncogene family, protein B (avian)
	Maf (*c-maf*)	NM_001025577.2	avian musculoaponeurotic fibrosarcoma (v-maf) AS42 oncogene homolog
	Myd88	NM_010851.2	myeloid differentiation primary response gene 88
	Kdm4a (*Jmjd2a*)	NM_172382.2	lysine (K)-specific demethylase 4A
	Ddr2	NM_022563.2	discoidin domain receptor family, member 2

Table S2

Expression levels of selected macrophage genes in the reprogrammed cells and BM derived macrophages as evaluated by NanoString technology. For each construct values represent two to three independent experiments. Value “1” substitutes for expression below the background level.

	Uninfected		MSCV		LAP		LAP*		
Gene symbol	#1	#2	#1	#2	#1	#2	#1	#2	#3
Tnf	112.7	1	1	85.3	5453.3	1	2039.1	1937.8	1817.5
Il1b	1	1	1	1	15567.5	11579.5	2244.8	1466.8	1628.5
Il6	1	1	1	1	4334.7	1	75.3	13.9	1
Il12b	1	1	1	1	93.2	1	643	135.1	87.2
Il12rb1	1.6	1	1	1	466.1	1	550.3	554.2	445.9
Ccr7	1600.8	2276.5	1	2671.6	11465.9	1	6346.2	2701.5	5113.3
Cxcl9	1	1	1	1	1	1	1	1	1
Cxcl10	9.4	1	1	1	3775.4	6616.8	2635.8	1499.7	1958.1
Ccl2	1	1	1	1	179539.1	161285.7	7163	6019.5	6087.5
Mmp8	1	1	1	1	35376.5	35979.1	96186.3	83282.7	84473.4
Mmp9	1	1	1	1	512.7	1	640.1	2208	2278
Nos2	1	1	1	1	2377.1	1	1184.7	763.7	644.6
Il10	1	1	1	1	885.6	1	1	1	1
Arg1	1	1	1	1	34537.5	28121.6	136.1	256.3	872.4
Il13ra1	1	1	1	1	1817.8	1	15148.6	9964.4	11050.5
Msr1	1	1	1	1	18317.5	8271.1	7742.3	5422.1	7231.3
Tgfb1	18800	17493.4	31248.2	23816.6	20601.3	5789.7	21462.9	18126.1	23201.2
Il4ra	1629	2516.2	1	3012.6	24236.8	26053.8	15137	9347.9	11462.5
Alox15	1	1	1	1	1	1	1	1	1
Mmp12	1	1	1	1	1	1	289.6	503.9	542.8
Pparg	1	1	1	1	1	1	49.2	48.5	33.9
Ccl22	1	1	1	1	14821.8	1240.7	19354.3	10321.2	21509.7
Kdm6b	600.9	239.6	1	1193.7	512.7	1	3105	2675.5	3576.9
Chi3l3	1	1557.6	1	1	1	1	81098.5	142875.3	70796
Fcgr3	1	1	1	1	16639.5	21091.2	30876.5	23208.8	27960.7
Mafb	1	1	1	1	1	1	674.9	445.1	930.6
Maf	1	1	1	1	4334.7	3308.4	330.2	207.8	416.8
Myd88	887.3	1677.4	1	1961	4241.4	1	5390.3	3960.5	4681.9
Kdm4a	768.3	2156.7	1	2216.8	1	1	952.9	755	1274.7
Ddr2	1	1	1	1	1	1	1	1	1

	ΔCR1,2		ΔCR3			ΔCR4		
Gene symbol	#1	#2	#1	#2	#3	#1	#2	#3
Tnf	1749	1467.1	2146.6	1153.2	1949.9	1982.7	3211.4	3420.2
Il1b	347	1	847	288.3	580.4	1562.1	2768.4	1741.8
Il6	1	1	1	1	1	15	1	1
Il12b	1	1	1	1	1	1	1	1
Il12rb1	922	427.9	690	557.4	618.4	1028.9	664.4	855.1
Ccr7	266.2	1	2427.7	307.5	531.1	2838.9	442.9	1013.4
Cxcl9	1	1	1	1	1	1	1	1
Cxcl10	42.8	1	1296	384.4	481.8	706	1	63.3
Ccl2	2257.6	4360.5	9729	7649.4	12333.1	8952.3	4872.4	5795.4
Mmp8	10760.2	14202.1	165079.6	45281.5	85576.5	23154.4	43630.5	66092.7
Mmp9	1	1	766.6	634.2	1464.3	405.6	1439.6	2216.8
Nos2	1	1	500.1	115.3	318.7	1	1	1
Il10	1	1	1	1	1	1	1	1
Arg1	1	1	65.7	595.8	6179.8	195.3	1	1
Il13ra1	5860.1	6112.8	9663.3	2383.2	5087.2	3815.2	6644.2	7030.5
Msr1	5988.5	7539.1	11098	6803.8	11456.7	8103.7	14949.5	12825.8
Tgfb1	18664	22352.5	21005.9	14799.1	18990.9	16875.7	22147.5	25556.7
Il4ra	4833.5	5786.8	10897.2	4920.2	7340.7	5407.4	8637.5	6618.8
Alox15	1	1	1	1	1	292.9	1	538.4
Mmp12	1	1	773.9	1	273.1	90.1	332.2	253.3
Pparg	1	1	21.9	115.3	3.8	1	1	1
Ccl22	1596.9	1548.6	9623.2	3997.7	4419.6	7465.3	9191.2	5637
Kdm6b	979.1	794.7	2372.9	961	2219.3	1126.6	775.2	2660.2
Chi3l3	1	1	53077.1	77243.7	111058.3	42508.5	14063.6	45413
Fcgr3	6492.2	9739.7	29716.4	10724.6	18220.8	11047.7	13288.5	14219.3
Mafb	61.8	20.4	368.7	249.9	485.6	180.2	1	1
Maf	1	1	441.7	1	239	1	110.7	1
Myd88	2704.3	2404.4	4913.8	4939.4	4222.3	3852.8	8526.8	5003.7
Kdm4a	608.4	529.8	938.2	1134	1153.3	480.7	1218.1	1235.1
Ddr2	1	1	1	1	1	1	1	1

	ΔCR6			MPh WT			MPh KO		
Gene symbol	#1	#2	#3	#1	#2	#3	#1	#2	#3
Tnf	1370.9	1220	1514.4	7761.4	1073.8	1219.5	7363.6	3312	2107.9
Il1b	305.9	499.1	115.1	1193	256	1	69.5	1	35.8
Il6	1	1	1	1	1	1	1	1	1
Il12b	1	1	1	1	1	1	1	1	1
Il12rb1	696	457.5	451.1	1	1	1	1	3.3	1
Ccr7	678	464.4	220.9	1	1	1	2171.1	1	1
Cxcl9	1	1	1	1	1	1	1	6.7	27.8
Cxcl10	733.1	519.9	138.1	774.7	466.2	115.8	4636.3	813.8	942.6
Ccl2	4580.2	2620.2	5224.5	9719.6	23794.7	15413.3	15556	10317.8	6108.9
Mmp8	51888.4	42553.8	51550	6169.2	8036	13761.6	4000	5318	3758.4
Mmp9	151.4	672.4	731.9	118.8	214	872.2	246	1142	1328.4
Nos2	402.2	305	174.9	1	26.7	1	1	1	1
Il10	1	1	1	2143.5	221.6	216.1	1	1	1
Arg1	34.1	159.4	87.5	1	1	1	219.2	271.3	127.3
Il13ra1	4876	4159	4483.4	884	1238.1	2415.8	3262	1306.1	1622.7
Msr1	6344.3	4796.7	6959.9	26844	33512.1	50106.7	53069	49311.8	47193.2
Tgfb1	15547.8	14646.7	18076.3	32110.2	37795.7	49921.5	31887.4	38843.3	36729.2
Il4ra	5215	5178	5542.1	10893.5	7481.9	9616.9	7010.6	6041.3	5234
Alox15	1	1	1	1	1	1	1	1	1
Mmp12	97.3	1	23	337.5	1112	4036.6	2609.6	3713.9	4188
Pparg	71.2	13.9	115.1	1031.4	2159	2477.6	1283.4	1419.9	1332.4
Ccl22	3822	2467.7	2375.2	1	1	1	7171	67	103.4
Kdm6b	1590.6	1774.5	2154.2	2029.5	554.1	532.6	1246	428.7	564.8
Chi3l3	65761.4	79791	105373.9	1311.8	107	2570.2	5502.6	1624.2	71.6
Fcgr3	14594	11562.1	14380	20750.9	44200	67727.5	25486.4	38679.2	36959.9
Mafb	73.2	34.7	267	31815.5	38170.1	56945.1	22267.1	26871.2	27649.3
Maf	55.2	1	78.3	6473.4	6969.9	7733.7	1513.4	4182.7	3161.9
Myd88	2859.2	3958	3461.5	4116	4581.6	6244	3839.5	4547.7	4207.9
Kdm4a	595.7	1150.7	980.5	779.5	871.2	1211.8	1112.3	1165.4	1113.6
Ddr2	1	1	1	38	145.2	1	1	3.3	1

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Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig und nur unter Verwendung der angegebenen Hilfsmittel angefertigt habe. Diese Arbeit wurde keiner anderen Prüfungsbehörde vorgelegt. Die Promotionsordnung der Mathematisch-Naturwissenschaftlichen Fakultät I der Humboldt-Universität zu Berlin habe ich gelesen und akzeptiert.

Berlin, 09.08.2012

List of publications and conference presentations

EMBL Conference “Stem Cells in Cancer and Regenerative Medicine”, 29 August - 1 September 2012, EMBL Heidelberg, Germany
Poster presentation “Structural plasticity and post-translational modifications of C/EBPβ determine the outcome of B cell to myeloid reprogramming“, Stoilova, B., Kowenz-Leutz, E., Scheller, M. and Leutz, A.

Conference “Stem Cells in Development and Disease”, 11 - 14 September 2011, Max Delbrück Center for Molecular Medicine, Berlin, Germany
Poster presentation “C/EBPβ domains involved in B cell to myeloid cell reprogramming”, Stoilova, B., Kowenz-Leutz, E., Scheller, M. and Leutz, A.

EMBO Conference “Advances in Stem Cell Research: Development, Regeneration and Disease”, 6 - 8 April 2011, Institut Pasteur, Paris, France
Poster presentation “C/EBPβ isoforms and domains involved in B cell to myeloid reprogramming”, Stoilova, B., Kowenz-Leutz, E., Scheller, M. and Leutz, A.

5th Annual Meeting of the German Society for Stem Cell Research (GSZ), 30 September - 2 October 2010, Lübeck
Poster presentation and abstract publication “B cell to myeloid reprogramming by the transcription factor C/EBPβ”, Stoilova, B., Kowenz-Leutz, E., Scheller, M. and Leutz, A., JSRM/Vol6 No.2, 2010; p84

9th International Plant Molecular Biology Congress, 2009, St. Louis, USA
Abstract “Genomic and gene-specific repair of UV-C induced DNA damage in barley (*Hordeum vulgare* L)”, Manova V.I., Borisov B. P., Stoilova B.D., Gecheff K.I. and L. M. Stoilov.

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